UNCOVERING THE CIRCADIAN OUTPUT PATHWAYS

OF *Neurospora crassa*

A Dissertation

by

MICHAEL WILLIAM VITALINI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

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Approved by:

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ABSTRACT

Uncovering the Circadian Output Pathways

of *Neurospora crassa*. (May 2007)

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The ubiquity of circadian systems has allowed their characterization in a broad range of model systems, which has greatly improved knowledge of how these systems are organized and the vast range of cellular and organismal processes under circadian control. Most of the advances, however, have come in describing the central oscillators of these systems, and, in some cases, the input pathways used to coordinate these oscillators to external time. Very little progress has been made in understanding the output pathways that allow circadian systems to regulate the breadth of processes shown to be clock-controlled.

A genetic selection was designed to obtain mutations in genes involved in circadian-regulated expression of the *Neurospora crassa ccg-1* and *ccg-2* genes. Some, but not all, of the strains obtained display altered regulation of more than one ccg as well as an 'Eas-like' appearance on solid media, and altered circadian period on race tubes. The data suggest a model in which output from the clock to these two genes is through a single, bifurcated pathway.

The cloning of the gene mutated (*rrg-1*) in one of the strains from the above selection led to the first molecular description of a circadian output pathway in *Neurospora*, the HOG MAP kinase pathway. The HOG pathway has been previously described with regard to its role in the osmotic-stress response. The discovery of the involvement of *rrg-1* in circadian regulation of *ccg-1* and *ccg-2* led to the discovery of regulation of the HOG pathway by the circadian clock. The data indicate that osmotic stress information and time-of-day information are transduced through the HOG pathway and implicate a role for the clock in preparing the organism for daily occurrences of hyperosmotic stress associated with sun exposure.

The genetic selection, and the description of the HOG pathway with regard to circadian output, provide a basis for further characterization of circadian output in *Neurospora*. The ubiquity of MAP kinase pathways, such as the HOG pathway, and the observed similarities in the mechanisms of circadian clock function across multiple phyla, indicate that these findings may well be applicable to other model systems.

ACKNOWLEDGMENTS

I would like to thank the members of the Bell-Pedersen lab, both past and present, for their continuous help, input, and support, and the occasional beer. I would most also like to thank my committee: Drs. Vincent Cassone, Daniel Ebbole, and Susan Golden, for their support and advice over the years. I especially thank Dr. Deborah Bell-Pedersen for her guidance, support, and patience; I could not have done this without all three. Finally, I would like to thank all of my friends, family and teachers that have inspired me to strive to accomplish more than I ever thought I was capable of.

NOMENCLATURE

ACE	activating clock elemant
Arg	arginine
bd	*band*
BMAL-1	brain and muscle ARNT-like protein-1
C-box	clock-box
C-terminus	carboxy-terminus
CAMK-1	calcium/calmodulin dependent protein kinase-1
ccg	clock-controlled gene
cDNA	complemetary DNA
CikA	circadian input kinase
CK	casein kinase
cmt	copper metallothionein
CT	circadian time
DD	constant darkness
DNA	deoxyribonucleic acid
EST	expressed sequence tag
FA medium	FPA + anthrinillate containing medium
FAD	flavin adenine dinucleotide
FFC	FRQ/FRH complex
FLO	FRQ-less oscillator

FPA	p-fluorophenylalanine
FRH	FRQ-interacting RNA helicase
FRP	free-running period
frq	*frequency*
FWD-1	F-box WD-40 repeat containing protein-1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
grg-1	*glucose repressible gene-1*
HK	histidine kinase
HPT	histidine phosphotransferase
kb	kilobase
l-FRQ	long-FRQ
LD	light/dark
LdpA	light-dependent period
LL	constant light
LOV	light oxygen voltage
LRE	light response element
MAPK	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
mtr	*methyltryptophan resistant*
N-terminus	amino terminus
NAD	nicotinamide adenine dinucleotide
ND	no data

ORF	open reading frame
PAS	PER-ARNT-SIM
ROS	reactive oxygen species
RR	response regulator
s-FRQ	short-FRQ
Trp	tryptophan
TA medium	Trp/Arg containing medium
wc	*white collar*
WCC	WHITE COLLAR complex
WT	wild type
ZT	zeitgeber time

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CHAPTER I

INTRODUCTION*

A Brief History of Circadian Research

The rotation of the Earth about its axis imparts a predictable daily cycle in environmental factors with which organisms living on or near the surface must cope and/or utilize. The significance of this daily cycle is such that nearly every species has evolved a means to anticipate and prepare for the resulting variations. Circadian (from the Latin *circa*, about; and *diem*, a day) clocks are biological systems that measure the passage of time and confer daily rhythms in endogenous processes that control an organism's internal biology, physiology, and behavior. This endogenous clock is synchronized to the cyclic external environment to entrain its oscillation to the 24 hour day.

Circadian clocks exist at all levels of life from the unicellular prokaryotic cyanobacteria to humans, and everything in between. Within these organisms, circadian systems regulate rhythms at many different levels including gene expression, hormone production, and even the sleep/wake cycles of an entire organism. The ubiquity of circadian systems, both in terms of their presence within virtually all organisms, as well

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This dissertation follows the style of Genetics.

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as the biological processes under circadian control, has only recently begun to be recognized.

A French astronomer by the name of Jean Jacques d'Ortuos de Mairan is credited with performing the first experiment in chronobiology (*i.e*. biological rhythms). In 1729, de Mairan noticed that the leaves of a heliotrope plant continued to remain open during the daylight hours, and closed during the night, even when the plant was isolated from the daily light/dark (LD) cycle thought to drive such rhythms (de Mairan, 1729; Somers, 1999). At that time, and for many years after, most believed that the continuing rhythm of leaf movement, and other rhythms described in biological systems, were a result of some environmental cue that was either uncontrolled for (such as gravity) or unidentified (Somers, 1999).

Although he did not recognize it at the time, another Frenchman, Augustin de Candolle, was among the first to demonstrate the truly endogenous nature of the plant circadian rhythm. One hundred years after de Mairan's discovery, Candolle reported that the period (the time needed to complete one full cycle) of the rhythm of leaf movement in the mimosa plant was, in fact, not exactly 24 hours, but rather 22-23 hours in constant darkness (DD; Somers, 1999). This would not be the case if the rhythm was being driven by an environmental cycle resulting from the 24-hour rotation of the Earth. The importance of this result would also go unrecognized for another 100 years.

In 1932 a German scientist named Erwin Bünning identified two variants of a common bean plant whose endogenous periods differed by three hours (Somers, 1999). Progeny from a cross of these two variants displayed periods that spanned the gap between the two parental extremes, demonstrating the polygenic control of period length in these plants (Somers, 1999). This experiment, and others, finally began to shed light on the issue of the exogenous versus endogenous nature of circadian rhythms; however, the debate would continue for years to come. Bünning was soon joined in his chronobiological endeavors by two other founding fathers of the field, Jürgen Aschoff and Colin S. Pittendrigh.

Bünning, Aschoff, and Pittendrigh laid the foundation for the modern field of chronobiology. Their pioneering works and ideas led to the description of circadian rhythms in quantitative terms, thus lending validity to their study. These rhythms were no longer just a curiosity that was observed in some species of plants; they became a *bona fide* biological phenomenon worthy of study. Throughout the 1950s, these three men, and their colleagues, created the infrastructure of chronobiology. Circadian rhythms were sought out, discovered, and described in many organisms, including *Gonyaulax, Neurospora, Drosophila,* and mice (Pittendrigh, 1993). In 1960 the first biological clocks conference was held at Cold Spring Harbor. This conference helped pave the way for the study of circadian rhythms to become a field of science in and of itself that has grown in leaps and bounds ever since.

General Characteristics of Circadian Rhythms

There are three cardinal properties that distinguish biological rhythms deemed to be generated by an endogenous circadian clock from those that are purely driven by environmental cues. First, the periods of circadian rhythms are, by definition, close to 24 hours in period length. Generation of that period is endogenous to the organism and self-sustaining, such that it occurs in the absence of environmental cues and is not simply driven by external stimuli. The period exhibited in constant conditions is termed the free-running period (FRP) and its exact value can vary between species, among individuals within a species, and slightly within an individual organism depending on the environmental conditions such as light intensity. Second, the rhythms are synchronized by, and entrainable to, environmental signals such as light and temperature. In order to maintain synchrony, circadian systems must be able to sense and respond to external stimuli in order to continually reset the internal clock to match the outside world. Thirdly, circadian rhythms are temperature compensated such that the period length of the rhythm is relatively constant at different physiologically-relevant temperatures. This property is in contrast to most biochemical reactions, which double in rate with a 10ºC temperature increase. It is important to note that the rhythms are not temperature insensitive, as temperature can be as strong or stronger a '*zeitgeber*' (literally 'time-giver') as light in entraining biological clocks.

The ubiquity of circadian systems has allowed for their characterization in a broad range of model organisms. Strikingly, while the individual genes and proteins can vary among

un-related organisms, the overall organization and underlying mechanisms of each clock system appear highly conserved (Bell-Pedersen et al., 2005). At the core of every biological clock system is an autonomous, entrainable oscillator. These oscillators are comprised of both positive and negative elements with opposing functions: a positive effector protein(s) stimulates expression of the negative effector(s) which acts to inhibit the activity of the activating positive effector. The net affect is an autoregulatory, negative-feedback loop in which the negative element ultimately inhibits its own expression. The interplay of these elements results in an oscillation with a period near 24 hours. Input pathways feed time-of-day information from the environment into the oscillator so that it is synchronized to local time. Output pathways relay the time-of-day information from the oscillator to downstream effectors, thus ensuring that specific processes occur at the proper time of day.

Circadian Rhythms in *Neurospora crassa*

Before circadian rhythms research became firmly established in the 1950s, *Neurospora crassa* (hereafter *N. crassa*) had been well-known as a premier model organism for genetics and biochemistry. In fact, it was the biological system used by Beadle and Tatum in developing their famous 'one gene, one enzyme' theory in the 1940s (Beadle and Tatum, 1941). In 1953, a pattern in the growth of *N. crassa* during LD cycles or DD was reported (Brandt, 1953); cultures grown in either of these conditions alternated between vegetative (mycelial) growth and differentiated aerial hyphae, which form conidia. In 1959, Pittendrigh and colleagues published the first paper demonstrating that

this growth pattern is controlled by an endogenous, temperature-compensated biological clock, and *N. crassa* remains a leading model organism for the study of circadian rhythms to this day. The following is a brief summary of the events and people that have led to our current understanding of the *N. crassa* circadian system.

As mentioned above, the first demonstration that *N. crassa* possesses a biological clock occurred in the late 1950s (Pittendrigh et al., 1959). Seven years later, the *band* (*bd*) mutation was isolated and conditions for persistent conidiation rhythms in constant conditions (the race tube assay; Figure 1-1 for description) were defined (Sargent et al., 1966). While the rhythms reported prior to use of the *bd* mutation continued for some time in constant conditions, they had a tendency to damp (decline in amplitude) over the course of an experiment due to a build up in CO_2 levels in the growth tube. Passing sterile air through the tubes alleviated this problem, but more conveniently, the *bd* mutation confers resistance to the elevated CO_2 levels without affecting the underlying circadian rhythm (Sargent and Kaltenborn, 1972). For this reason, *bd* is still used as the laboratory pseudo-wild-type strain for circadian rhythms research.

One of the scientists who worked with Colin Pittendrigh to demonstrate the circadian nature of the *N. crassa* conidiation rhythm was Victor Bruce. The year after the *bd* strain was isolated, the first mutations affecting period length in *Neurospora* were obtained by one of Bruce's former students, Jerry Feldman (Feldman and Hoyle, 1973). Although

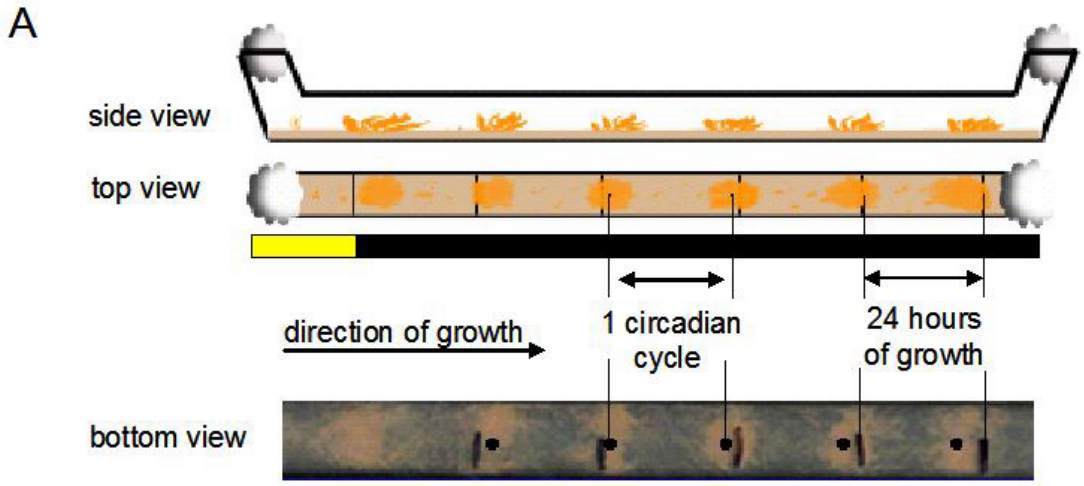

Figure 1-1. Description of the race tube assay and definition of circadian terminology. (A) Cartoon schematic and picture of an actual race tube. Race tubes are hollow glass cylinders approximately 30 cm in length and bent at the ends in order to accommodate an agar medium. Conidia are inoculated at one end of the tube and the culture is allowed to germinate and grow in constant light (LL) conditions for ~24 hours, after which time the growth front is marked on the tube and the culture transferred to constant dark (DD). This LL to DD transfer synchronizes the cells to dusk. Every 24 hours after the transfer to DD, the growth front is marked under a red safe light (*N. crassa* does not respond to illumination by red light). Once a day (every 22 hours in wild-type *bd* strains) the circadian clock initiates the asexual development program resulting in the production of a 'band' of fluffy orange conidiospores. At the end of an experiment, the race tube acts as a 'fossil record' of the circadian rhythm and can be analyzed at leisure. Period is determined by calculating the distance between bands of conidiospores as compared to the distance between the 24 hour growth-front markings.

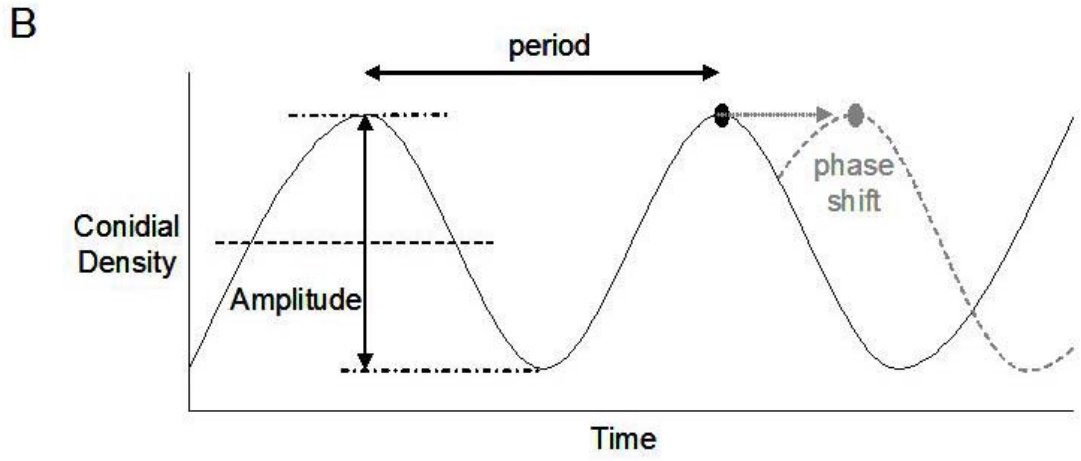

Figure 1-1 Continued.

(B) Sample graph of circadian variables and terminology. Race tubes are frequently scanned to create digital images which can be used to generate plots representing the conidial density over the course of an experiment. From these plots one can obtain period, amplitude and phase information. The period of a rhythm is the amount of time required for one complete cycle to occur. Amplitude is the distance from the midline of the curve to either the peak or the trough position and is representative of the robustness of the rhythm. Phase is an arbitrary point on the curve with reference to some point in time (such as the transition to DD). The application of a stimulus (*i.e.* light or temperature) can 'phase shift' a circadian rhythm such that the point of interest (in this case the peak of conidial density) occurs prior to (phase advance) or after (phase delay, pictured) the time at which it would be predicted to occur in the absence of said stimulus.

Ron Konopka and Seymour Benzer were the first to isolate a gene associated with a circadian system (the *period* gene of *Drosophila*; Konopka, 1971), Feldman's discovery turned out to be no less significant. The mutations he obtained, which he dubbed *frequency* (*frq*), resulted in FRPs ranging from 16.5 to 29 hours (Feldman and Hoyle, 1973; Gardner and Feldman, 1980; the wild-type FRP in *N. crassa* is about 22 hours). During his characterization of the *frq* mutant strains, Feldman also made the observation that, for each different allele of *frq*, the period length was changed by a multiple of 2.5 hours (Gardner and Feldman, 1980), a phenomenon that has defied explanation to this day. Some of these mutations (*i.e.*, frq^7) were also found to have an effect on temperature compensation of the clock (Gardner and Feldman, 1981). It was later discovered that the *frq* locus encodes a key component of the core *N. crassa* circadian oscillator (Aronson et al., 1994a).

Characterization of the *N. crassa* clock and its overt rhythms proceeded slowly during the 1980s. Much effort was focused on describing the physiological basis of the clock, and the effects of various drugs and chemicals were examined (Nakashima, 1982, 1986; Schulz et al., 1985). Cultures of *N. crassa* were even taken into outer space in order to definitively show that the observed rhythm is not a result of some exogenous cue resulting from the Earth's rotation (Ferraro et al., 1989; Sulzman, 1984; Sulzman et al., 1984), but little progress was made in determining the true nature of the molecular oscillator.

In the late 1980s the next generation of *N. crassa* chronobiologists emerged on the scene equipped with many of the molecular biology techniques used today. Jay Dunlap and Jennifer Loros (former students of Jerry Feldman) have dissected the molecular workings of the circadian oscillator in *N. crassa*. Their work laid the foundation for the current *N. crassa* chronobiologists, many of whom have worked in the Dunlap lab during their career, and each of whom has carved their own niche in the field.

In 1989, the first two clock-controlled genes (ccgs) were identified through a subtractive hybridization assay (Loros et al., 1989). By 1991 it was demonstrated that the clock regulates these genes at the transcriptional level (Loros and Dunlap, 1991), which provided the first insight into a mechanism for clock-controlled gene expression. Until 1996, however, it was thought that the clock in *N. crassa* may only serve to regulate development; the most robust rhythm observed was that seen in conidiation, and both ccgs isolated up to that point were also induced in response to light and development (Arpaia et al., 1995; Bell-Pedersen et al., 1992). A new, more rigorous screen isolated six more ccgs, among them was the previously cloned copper metallothionein gene (*cmt*) involved in copper transport and detoxification (Bell-Pedersen et al., 1996b). In addition to *cmt,* two of the other new-found ccgs were shown not to be induced during conidiation. This discovery marked the first evidence that the *N. crassa* clock regulates processes other than development.

A more complete picture of the central oscillator began to come together in 1997. Studies thus far had implicated a regulatory feedback loop in the generation of circadian

oscillations (Dunlap, 1996); however, only negative elements of such a loop had been identified (*frq* in *N. crassa*, and *per* and *tim* in *Drosophila*). It had been known for some time that the conidiation rhythm was responsive to light (Sargent and Briggs, 1967), and that the *wc-1* and *wc-2* genes and protein products were required for this, as well as all other, known light responses in *N. crassa* (Russo, 1988). Initially, it was assumed that inability of *wc* mutant cells to perceive light prevented the synchronization of their oscillators, and, therefore, cultures of these strains appeared arrhythmic. The turning point came when Susan Crosthwaite (in the Dunlap lab at the time) demonstrated that the products of both *wc-1* and *wc-2* are necessary for robust expression of *frq* transcripts, even in dark-grown cultures (Crosthwaite et al., 1997). This finding marked the identification of the first 'positive elements' in any circadian system.

The FRQ/WCC Oscillator

We now know that these three genes (*frq, wc-1*, and *wc-2*) and the interactions of their protein products comprise a core circadian oscillator in *N. crassa.* Deletion of any one of these genes results in a loss of circadian regulation of conidiation (Aronson et al., 1994a; Collett et al., 2002; Crosthwaite et al., 1997; He et al., 2002; Lee et al., 2003). Mutations in *frq* or the *wc* genes result in a variety of phenotypes, including altered period length (from 16 – 35 hours), arrhythmia, and/or affect temperature compensation of the circadian rhythm (Aronson et al., 1994a; Collett et al., 2001; He et al., 2005b; Liu et al., 2000; Yang et al., 2002). Constitutive expression of *frq* mRNA, which normally accumulates with a period equal to that of the conidiation rhythm, results in arrhythmia.

Also, it has been demonstrated that the clock can be phase shifted through manipulation of *frq* mRNA and protein levels (Aronson et al., 1994a; Crosthwaite et al., 1995; Huang et al., 2006; Liu et al., 1998), implicating FRQ as a necessary state-variable that determines both the period and phase of the oscillation.

The *N. crassa* circadian FRQ/WCC (see below) oscillator has the basic signature features of oscillators in other model systems, including *Drosophila* and mouse (Bell-Pedersen et al., 2005), and is depicted in Figure 1-2. In this oscillator, both *frq* mRNA and FRQ protein levels are low at subjective dawn. An increase in the levels of *frq* mRNA occurs when WC-1 and WC-2 proteins heterodimerize through their PER-ARNT-SIM (PAS) domains (forming a WHITE COLLAR complex, WCC) and directly activate *frq* transcription by binding to the "Clock box" (C-box) in the *frq* promoter (Cheng et al., 2002; Denault et al., 2001; Froehlich et al., 2003a). The accumulation of *frq* transcript reaches its peak about four to five hours later, leading to an increase in the levels of FRQ protein. FRQ protein enters the nucleus, homodimerizes, and interacts with FRH (a FRQ-interacting RNA helicase) to form the FRQ/FRH complex (FFC; Cheng et al., 2005; Cheng et al., 2001a). The interaction of the FFC with the WCC promotes the phosphorylation of the WCC by casein kinase I (CK I) and CK II (He et al., 2006). Hyperphosphorylated WCC is inactive and unable to support transcription from the *frq* promoter (He et al., 2006; He et al., 2005b). For the remainder of the day and into the early evening, the FFC remains at sufficient levels to promote

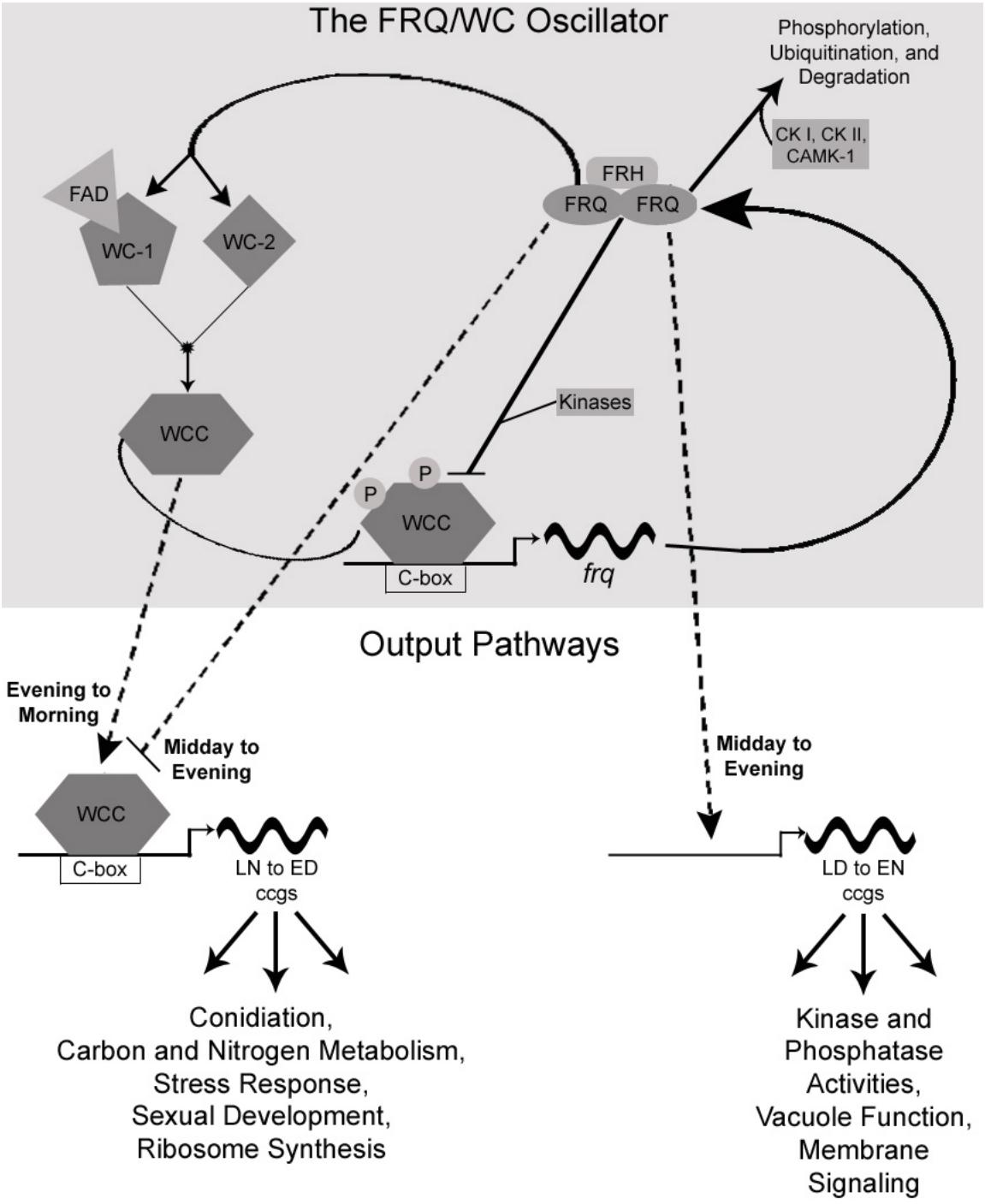

Figure 1-2. Cartoon model of the FRQ/WCC oscillator and output pathways. The model of the FRQ/WCC oscillator is drawn within the shaded area with solid lines connecting aspects of the oscillatory loop. Details of the model are included in the text. Other oscillators are omitted for simplicity. P = phosphorylation.

Figure 1-2 Continued.

A squiggly line represents rhythmic transcript accumulation, and arrows depict activation, while bars indicate repression. The WCC, which peaks in accumulation in the late evening to early morning, is also thought to function in an output pathway to directly turn on the transcription of ccgs that contain a consensus C-box promoter element (dashed line at left). These primary ccgs would be predicted to include select members of the ccgs that peak in the late night (LN) to early day (ED). Some of these primary targets of the WCC likely include transcription factors and signaling components that would function to activate the expression of downstream ccgs that lack the C-box, including the terminal ccgs responsible for overt rhythms in development, metabolism, stress responses, *etc*. The model also includes speculation that FRQ can function in the output pathways (dashed lines at center and right), either by inhibiting the WCC, and thus inhibiting the expression of C-box containing ccgs during the late day and early evening, or by directly or indirectly activating transciptional/posttranscriptional regulation of primary target ccgs. These ccgs would be predicted to peak in the late day (LD) to early night (EN), and similar to WCC primary target ccgs, would include effectors of downstream/terminal ccg activation.

phosphorylation of the WCC and thus inhibit transcription of *frq*. FRQ protein is progressively phosphorylated by CK I, CK II, and probably calcium/calmodulin dependent kinase-1 (CAMK-1; He et al., 2006; Yang et al., 2003; Yang et al., 2002; Yang et al., 2001). Hyperphosphorylated

FRQ interacts with FWD-1, an F box/WD-40 repeat containing protein and the substrate-recruiting subunit of an SCF-type ubiquitin ligase complex (He et al., 2005a; He and Liu, 2005a). FRQ is then ubiquinated and targeted for degradation by the proteosome pathway. Degradation of FRQ, in conjunction with dephosphorylation of the WCC by protein phosphatase 2A, releases the inhibitory effect on the WCC and leads to reactivation of *frq* transcription, allowing the cycle to start anew (Liu et al., 2000; Schafmeier et al., 2005). FRQ also plays a role in the positive regulation (either directly or indirectly) of WC-1 and WC-2: FRQ influences the steady state levels of *wc-2* mRNA, and FRQ up-regulates WC-1 through a post-transcriptional mechanism (Cheng et al., 2001b; Schafmeier et al., 2006). WC-1 levels cycle during the course of a day independent of FRQ, with peak levels occurring around subjective midnight (de Paula et al., 2006; Lee et al., 2000), but *wc-1* and *wc-2* mRNA and WC-2 protein levels do not appear to accumulate rhythmically. The mechanism(s) for the positive regulation of WC-1 and WC-2 by FRQ is not known; however, the positive feedback loops are important for maintaining the robustness and stability of the developmental rhythm (Cheng et al., 2001b; Lee et al., 2000; Schafmeier et al., 2006).

Time-of-day information from light cues is received directly by the FRQ/WCC oscillator. WC-1 bound to a flavin (FAD) acts as a blue light photoreceptor that together with its partner, WC-2, forms a multimeric complex (that differs in composition from the complex formed in the dark; He and Liu, 2005b) and binds light-response elements (LREs) in the *frq* promoter to activate *frq* transcription (Froehlich et al., 2002; He et al., 2002). The net effect of this activation is a change in the levels of FRQ protein and, thus, a change in the phase of the rhythm. The mechanism by which temperature information is perceived by the FRQ/WCC oscillator is not clear, but it also involves changes in the relative levels in FRQ protein. Absolute levels of FRQ are higher at warm temperatures and lower at cooler temperatures (Figure 1-3; Liu et al., 1998). Upon a temperature step-up, the current level of FRQ protein becomes the trough level (equivalent to dawn) of a new cycle; upon a temperature step-down, the current level of FRQ becomes the peak level (equivalent to dusk) of a new cycle, thus resetting the clock to a new phase in both instances (Figure 1-3; Liu et al., 1998; Bell-Pedersen, 2000).

There are two different isoforms of FRQ protein, long (l-FRQ) and short (s-FRQ), and in addition to changes in the absolute levels of FRQ, temperature also affects the ratio of these forms. This ratio between the two forms is necessary to support rhythms over a broad range of temperatures: strains engineered to express only s-FRQ are rhythmic at low, but not high temperatures, and strains that only express l-FRQ are rhythmic at high, but not low temperatures (Liu et al., 1997). The same *frq* transcript encodes both forms of FRQ protein and temperature-sensitive alternative splicing events determine the

relative levels of each form (for review see Brunner and Diernfellner, 2006). Briefly, the start codon of l-FRQ is contained within an intron (intron 6) with non-consensus splice sites. At lower temperatures this intron is spliced much more efficiently than at elevated temperature, which results in translation starting from the second, downstream start site (producing s-FRQ) and reduces the production of l-FRQ (Liu et al., 1997). At higher temperatures, the recognition of the non-consensus splice sites of intron 6 is much less efficient, which leaves the l-FRQ start codon in place and results in increased production of l-FRQ (Liu et al., 1997). The end result of these alternative splicing events is that the ratio of l-FRQ to s-FRQ increases with an increase in temperature, conferring rhythmic behavior over a broad range of temperatures.

Output from the FRQ/WCC oscillator is poorly understood and there has not yet been a description of a complete circadian output pathway. However, it is possible that components of the FRQ/WCC oscillator can directly transmit temporal information through output pathways, or through other undescribed oscillators, to control the ccgs and overt rhythmicity (Figure 1-3). FRQ has not been shown to directly bind DNA to regulate transcription, but it does function in both positive and negative regulatory pathways within the FRQ/WCC oscillator (reviewed in Dunlap and Loros, 2004; Brunner and Schafmeier, 2006; Liu and Bell-Pedersen, 2006). Thus, it is reasonable to speculate that FRQ can act on its own, or with partners, outside of the molecular circadian feedback loop to control rhythmic ccg expression.

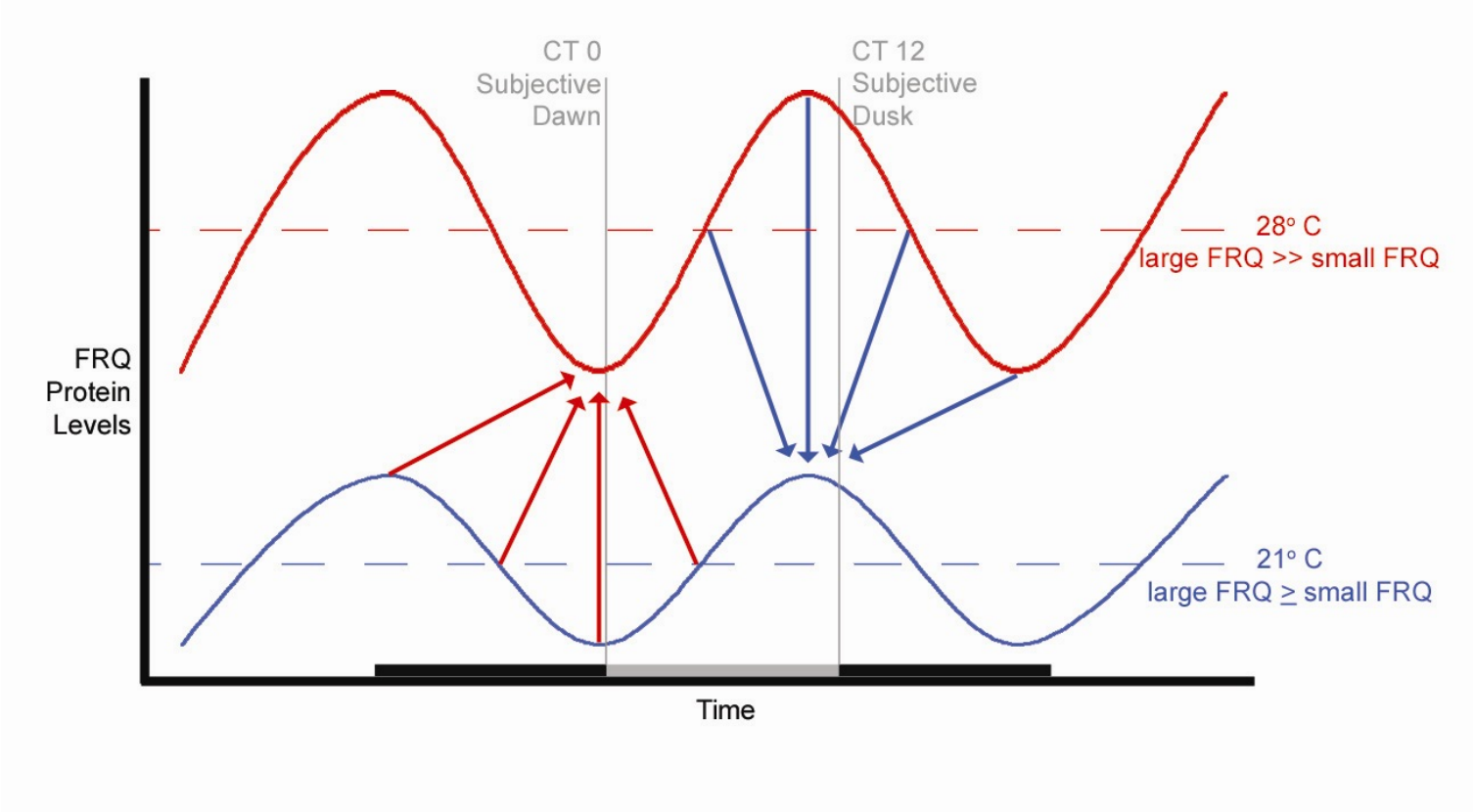

Figure 1-3. Temperature input to the FRQ oscillator. A cartoon graphic of relative levels of FRQ protein at 28ºC versus levels at 21ºC is shown. The dashed lines represent the average levels of FRQ protein, red arrows indicate an increase in temperature, blue arrows indicate a decrease in temperature. The black and grey bar represents time in DD. See text for details

Both WC proteins are PAS domain-containing transcription factors with GATA-type Zn-finger DNA-binding domains (Ballario et al., 1998; Ballario et al., 1996). WC-1 and WC-2 heterodimerize through the interactions of their PAS domains to form the WCC, which binds to specific promoter elements of light-responsive and clock genes to activate transcription (reviewed in Dunlap and Loros, 2004). Therefore, it is likely that the WCC is involved both in oscillator function and in direct output signaling by regulating transcription initiation of target ccgs. Similar to its negative role in the oscillator, the FFC may repress the transcriptional activation activity of the WCC in the output pathways. Interestingly, both WC proteins are post-translationally modified by phosphorylation and these modifications regulate their activity in the positive feedback loop (reviewed in Brunner and Schafmeier, 2006; Liu, 2003; Schafmeier et al., 2006). Two forms of WC-1 are expressed in cells (de Paula et al., 2006) which raises the possibility that different forms of the WCC, and their varying levels of phosphorylation, may mediate oscillator versus output functions.

Circadian Output Pathways and ccgs – An Overview

The ultimate function of the circadian clock is to regulate the levels and activity of target proteins to the most advantageous times of day. In eukaryotes, this regulation can take place at several levels, including the initiation of transcription, RNA processing and transport to the cytoplasm, mRNA stability, translation initiation, protein folding, protein processing (modification) and transport to the proper cellular destination, allosteric regulation, and protein stability. To date, most studies of clock regulation have

concentrated on aspects of the first potential control point, the regulation of transcript abundance, and little information exists for the others (for examples of post-transcriptional regulation see Heintzen et al., 1997; Mittag, 2003; Newby, 1996).

In many circadian systems, core circadian oscillator components are themselves transcription factors that are active in the feedback loop at specific times of day and recognize defined sequence elements in the promoters of their target genes: WC-1 and WC-2 in *N. crassa* (Froehlich et al., 2003b), CLOCK and CYCLE in *Drosophila* (Hardin, 2004), and CLOCK and BMAL1 in mammals (Bell-Pedersen et al., 2005; Hardin, 2004). These core oscillator components may be capable of directly regulating ccgs whose promoters contain the necessary promoter element(s). Alternatively, they may indirectly regulate ccgs by turning on the expression of a downstream set of control elements, such as transcription or signaling factors. While the dominant peak in ccg expression anticipates dawn or dusk in most organisms, clocks regulate genes at multiple phases of the day in all organisms and tissues examined (Bell-Pedersen et al., 2005). Thus, it is unlikely that all of an organism's ccgs are direct targets of core oscillator components. A more reasonable assumption is that both direct and indirect activation of the ccgs through the output pathways are responsible for their rhythmic transcription and phase differences.

Questions of what and how much of the genome is regulated by the clock at the level of transcript abundance in eukaryotic organisms have been addressed recently using microarrays to globally screen for ccgs (Duffield, 2003; Harmer et al., 2001; Panda and

Hogenesch, 2004). In eukaryotic model organisms, it appears that somewhere between 2 to 15% of the genome is regulated by the clock at the transcriptional level. However, this is very likely an underestimate if one considers the limitations of microarrays (such as detecting low amplitude rhythms in mRNA abundance) and clock-mediated post-transcriptional regulation that would not be detected from microarray studies. For example, in *Arabidopsis thaliana*, microarray experiments suggested that about 10% of the genome is clock-regulated at the level of transcript abundance (Harmer et al., 2000; Schaffer et al., 2001), whereas an enhancer trap experiment (which measures promoter activity) demonstrated that rhythmicity is more widespread (36% of the promoters were clock-controlled; Michael and McClung, 2003). Estimates of the extent of clock control of gene expression in eukaryotes differ from the prokaryotic cyanobacteria in which promoter trap experiments demonstrated that the clock globally regulates transcription (Liu et al., 1995). Studies in cyanobacteria have suggested that the regulation of chromosomal remodeling by the clock plays an important role in global clock control (Min et al., 2004; Smith and Williams, 2006; for review see Woelfle and Johnson, 2006). Regulation of chromosomal remodeling by the clock may also play a role in the regulation of transcription in eukaryotic cells (Doi et al., 2006; Etchegaray et al., 2003; Grimaldi et al., 2006; Ripperger and Schibler, 2006).

There is still much to be learned from the time-series microarray data for eukaryotic model systems; however, it is clear that the numbers and diversity of genes that are regulated by the clock in different organisms and tissues is vast. While there is evidence

for conservation of clock-controlled processes, including aspects of protein synthesis and processing, intermediary metabolism, chromatin modification, transcriptional regulation, and cellular signaling (Bell-Pedersen et al., 2005), current data reveal limited overlap for ccgs between different species. Thus, while the mechanisms of the molecular oscillators and input pathways appear to be conserved throughout evolution, many of the terminal products of the clock are not. This discrepancy may not be surprising as one role of the clock is to allow organisms to better adapt to a particular temporal niche, which would clearly translate into different requirements for different organisms. Rather, the ccgs that are conserved may include core mechanistic components of the output pathways. One caveat to microarray analyses is that a rhythm in mRNA accumulation may not necessarily lead to a rhythm in the corresponding protein if the protein is stable, and conversely, constant levels of mRNA may yield a rhythm in protein levels or activity (*e.g.*, Cheng et al., 2001b; Lee et al., 2000; Merrow et al., 2001). Therefore, efforts are needed to examine the proteomes of the model clock organisms at different times of the day in order to relate rhythmic protein levels to rhythmic mRNA levels.

What defines a ccg? In the past, standard criteria were used to define a rhythmically expressed gene as a ccg based on: 1) demonstration of rhythmic expression or activity under constant conditions, 2) demonstration that the period of the rhythm matched the genotype of the strain, and 3) demonstration that inactivation of the gene had no effect on the function of the oscillator itself (Loros et al., 1989; Loros and Dunlap, 2001). This last criterion would distinguish an oscillator component that displays a rhythm from an

output pathway component. However, this definition has become somewhat muddled as we learn more about the circadian clock as a system. For instance, examples of ccgs that feed back onto the oscillator or the input pathway have now been uncovered, which, when inactivated, affect the operation of the oscillator (Cassone et al., 1993; Gwinner et al., 1997; Heintzen et al., 2001; Heintzen et al., 1997; Herzog and Block, 1999; Pregueiro et al., 2006). Also, ccgs can be regulated by more than one oscillator, so that a mutation in a component of one oscillator might not necessarily affect the period of a ccg regulated by a different circadian oscillator (de Paula et al., 2006).

Control of Gene Expression by the *N. crassa* Circadian Clock

A variety of processes in *N. crassa* are known to be regulated by the clock, but the conidiation rhythm remains the best-characterized output of the FRQ/WCC oscillator. Other rhythms, including those of enzymatic activity, CO_2 evolution, and lipid metabolism, have been described (reviewed in Lakin-Thomas et al., 1990); however, these rhythms are more difficult to monitor and are not as well defined. Whether these other rhythms arise from output pathways of the FRQ/WCC oscillator or from other cellular oscillators is unknown. The discussion below of the output pathways in *N. crassa* focuses on clock regulation of mRNA levels. While aspects of posttranscriptional regulation are known for FRQ and the WC proteins, only rhythms in transcript abundance have been investigated with respect to the output pathways in *N. crassa*.

Table 1-1
N. crassa ccgs identified by methods other than microarrays

Gene	Average Peak[1]	Identity[2]	Devel.[3]	Light[3]	Reference
ccg-1	CT3	unknown	+	+	(Loros et al., 1989) (McNally and Free, 1988)
ccg-2	CT22	hydrophobin	+	+	(Loros et al., 1989) (Bell-Pedersen et al., 1992) (Lauter et al., 1992)
ccg-4	CT5	pheromone	+	+	(Bobrowicz et al., 2002) (Bell-Pedersen et al., 1996c)
ccg-6	CT19	unknown	+	+	(Bell-Pedersen et al., 1996c)
ccg-7	CT21	GAPDH	-	-	(Bell-Pedersen et al.,1996c), (Shinohara et al., 1998)
ccg-8	CT20	unknown	-	-	(Bell-Pedersen et al., 1996c)
ccg-9	CT19	trehalose synthase	+	+	(Bell-Pedersen et al.,1996c), (Shinohara et al., 2002)
cmt (*ccg-12*)	CT18	CuMT	-	-	(Bell-Pedersen et al., 1996c) (Munger et al., 1987)
ccg-13	CT0	unknown	ND	-	(Zhu et al., 2001)
ccg-14	CT0	unknown	ND	-	(Zhu et al., 2001)
ccg-15	CT4	unknown	ND	-	(Zhu et al., 2001)
lyz	CT2	lysozyme	ND	-	(Zhu et al., 2001)
al-3 [4]	CT10	GGPPS	+	+	(Arpaia et al., 1995)
con-6	ZT20	unknown	+	+	(Lauter and Yanofsky, 1993)
con-10	ZT20	unknown	+	+	(Lauter and Yanofsky, 1993)
vvd	CT3	light repressor	ND	+	(Heintzen et al., 2001)
bli-3	CT3	unknown	ND	+	(Eberle and Russo, 1994)
fl	CT3	developmental regulator	+	+	(Correa et al., 2002)
mfa	CT1	pheromone	ND	ND	(Bobrowicz et al., 2002)
prd-4	CT6	Checkpoint kinase-2	ND	ND	(Pregueiro et al., 2006)

[1] The peak in message accumulation can vary between experiments and culture conditions. The *con-6* and *con-10* mRNAs peak about 20 hours after a light pulse, representing zeitgeber time (ZT) 20.

[2] Abbreviations are as follows: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CuMT, copper metallothionein; GGPPS, geranylgeranyl pyrophosphate synthase.

[3] Developmental and light regulation of the ccgs. A + indicates increased transcript abundance following developmental induction by desiccation or light treatment, a - indicates no effect, and ND means no data.

[4]Only the *al-3*c transcript has been shown to be rhythmic.

To characterize circadian output pathways at the molecular level, genes that are rhythmically expressed under control of the clock were isolated (Table 1-1 and references therein). Twelve ccgs were identified through targeted approaches. In addition, eight genes being studied for other reasons were found to display rhythms in transcript accumulation with circadian periods. mRNA accumulation of these ccgs peaks in the late night to early morning. This timing coincides with initiation of conidiation, which suggests a potential role for these gene products in this developmental pathway. Indeed, the *ccg-2* gene encodes a component of the conidiospore (see below), and many of the other ccgs are also induced during development. Regulation of the ccgs by the FRQ/WCC oscillator has been verified for most of these genes by demonstrating that the period of the ccg mRNA abundance rhythm approaches the expected value of 29 h in the long-period frq^7 mutant strain. The FRQ/WCC oscillator functions normally in strains containing inactivated copies of the ccgs, demonstrating that the ccgs are not part of the central oscillator mechanism. Both *ccg-1* and *ccg-2* were directly shown to be controlled at the level of transcription initiation by nuclear run-on assays (Loros and Dunlap, 1991).

The clock responsive, light-responsive, and developmentally responsive *ccg-2* gene encodes a member of a class of low molecular weight, cysteine-rich, hydrophobic, secreted proteins called hydrophobins (Arpaia et al., 1993; Bell-Pedersen et al., 1992, 1996a; Loros et al., 1989; Wosten, 2001). Hydrophobins coat the outer cell wall of fungi and maintain the cell-surface hydrophobicity essential for air dispersal of mature conidiospores. The *ccg-1* gene is regulated by the FRQ/WCC oscillator and is also

induced by developmental cues (Arpaia et al., 1995; Loros et al., 1989). The function of *ccg-1* has remained elusive: *ccg-1*-null strains display no discernable phenotypes, and CCG-1 protein does not share homology with other known proteins outside of filamentous fungi. *ccg-1* is a glucose-repressible gene (*grg-1*; Lindgren, 1994; Loros et al., 1989; McNally and Free, 1988) and is induced by heat shock and hyperosmotic stress (discussed further in Chapter II), which suggests a role in general stress responses. *ccg-9* encodes trehalose synthase, which catalyzes the synthesis of the disaccharide trehalose; trehalose plays an important role in protecting cells from environmental stresses (Shinohara et al., 2002). Consistent with a role for *ccg-9* in stress responses, the transcript is induced by heat shock, glucose starvation, and osmotic stress. A role in development for *ccg-9* is also suggested by the finding that inactivation of *ccg-9* results in altered conidiophore morphology and abolishes the normal circadian rhythm of conidial development. *ccg-9*-null strains have normal FRQ cycling, phosphorylation, and light induction, indicating that loss of the conidiation rhythm may be a defect in circadian output and is not due to changes in either the FRQ/WCC oscillator or light input into the clock (Shinohara et al., 2002). *ccg-12* is the *N. crassa cmt* gene encoding copper metallothionein involved in copper detoxification (Bell-Pedersen et al., 1996b). The *con* genes are preferentially expressed during conidiation but are not essential for development (Berlin and Yanofsky, 1985). *con-6* and *con-10* are under circadian clock control, and *con-10* encodes a small stress-response protein of unknown function that is conserved in fungi. The *con-10* promoter has been studied in detail, and elements involved in its regulation by nitrogen or carbon starvation, heat shock, and light have

been identified (Lee and Ebbole, 1998). The circadian regulation of *ccg-1*, *ccg-9*, *cmt*, and *con-10* indicates that the clock plays a role in controlling stress responses in the organism. These genes, with the exception of *cmt*, are also induced during development. *ccg-7* encodes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis and gluconeogenesis, indicating that the clock can regulate aspects of intermediary metabolism (Shinohara et al., 1998). GAPDH is also rhythmic in several other phylogenetically distant organisms (*e.g.*, Bailey et al., 2003; Fagan et al., 1999; Greene et al., 2003), suggesting a conserved role for the clock in controlling key metabolic pathways. The "a-like" and "α-like" mating-type pheromone precursor genes, *ccg-4* and *mfa-1*, respectively, are also known to be clock-controlled (Bobrowicz et al., 2002). These data support a role for the *N. crassa* circadian clock in some aspects of mating and the sexual cycle. This link is consistent with data from several filamentous fungi demonstrating that sexual spore release is clock-controlled (Liu and Bell-Pedersen, 2006). This sampling of ccgs indicates that the FRQ/WCC oscillator plays an important role in the timing of sexual and asexual developmental processes, stress responses, and metabolism. Clock control of these genes may have evolved as a way to anticipate stresses (*e.g.*, increased mutagenic potential) and opportunities (*e.g.*, increased temperature) in the 24-hour LD cycle.

Genome-wide Screens for ccgs Using DNA Microarrays

The search for ccgs and an understanding of the role of the clock in *N. crassa* has recently been accelerated by the use of microarray technology. In one study, a gene set

was assembled from morning-specific and evening-specific expressed sequence tag (EST) libraries from cultures grown in the dark under starvation conditions (Nowrousian et al., 2003). cDNA microarrays of 1100 genes that were developed from these ESTs were used to examine transcript abundance from *bd;* from the long-period strain *bd*, frq^7; and from the *frq* knockout strain *bd,* frq^{10}. Twenty-seven genes were rhythmically expressed in *bd* grown in DD, including four of the previously known ccgs. As with the previously identified ccgs, the expression of all of these ccgs peaked in the subjective late night to midday and required *frq* for rhythmicity. Fourteen of the 27 genes responded to temperature cycles, and all 14 required FRQ for temperature-regulated expression. Thus, at least part of the temperature input pathway to the clock involves the FRQ/WCC feedback loop, in which *frq* RNA splicing may function as a temperature sensor (Colot et al., 2005; Diernfellner et al., 2005). Using a different set of cDNAs, microarrays were generated from EST libraries corresponding to the major stages of the *N. crassa* life cycle (conidial, mycelial, and sexual; Correa et al., 2003). These arrays contained 1343 genes (about 14% of the total estimated *N. crassa* genes) and had little overlap with the genes in the Nowrousian *et al.* (2003) study. The small degree of overlap, as well as the difference in the percentage of ccgs identified (5% of the transcriptome in the Nowrousian *et al.* [2003] study versus 20% in the Correa *et al.* [2003] study), may be due to different growth conditions: slowly starving cultures in Nowrousian *et al.* (2003) versus rapidly growing for Correa *et al.* (2003). In the Correa *et al.* (2003) study, 145 ccgs, including five previously known ccgs, had daily peaks in mRNA accumulation in *bd* and *bd, frq7* cultures. While most (119) of the genes peaked

in expression in the subjective late night to early day, 26 genes peaked between subjective noon and midnight and represent the first examples of *N. crassa* genes peaking in expression at times other than late night/early morning (Bell-Pedersen et al., 1996b). They indicate that mechanisms must exist for the oscillator(s) to regulate ccg transcript abundance at other times of the day. A new class of ccgs was also identified that cycled in a strain that lacks FRQ, providing molecular support for existence of a FRQ-less oscillator (FLO) in *N. crassa*.

The Functions of the ccgs Provide Insights into the Roles of the Clock in the Fungal Life Cycle

The predicted functions of the proteins encoded by the identified *N. crassa* ccgs are yielding further insights into processes that may be clock-regulated (Table 1-2). Recall that rhythmic RNA levels do not necessarily translate into rhythmic cellular functions, and only limited data exist in *N. crassa* to correlate rhythmic RNA to rhythmic activity of the encoded proteins (*e.g.*, see Garceau et al., 1997; Shinohara et al., 1998). With this qualification in mind, several genes encoding enzymes involved in carbon and nitrogen metabolism show circadian rhythms in mRNA accumulation, with peaks occurring in the late night to early morning. In addition, the genes encoding glycogen phosphorylase, mannitol-1-phosphate dehydrogenase, and a low-affinity glucose transporter peak in the early night, suggesting that flux into the glycolytic pathway may crest at this time of day to prepare for increased energy requirements related to the consequent development of conidiospores. These data further support a role for the *N. crassa* clock in controlling

Table 1-2

Functional classification of *N. crassa* ccgs

Functional Category[1]	Number of ccgs
Cell division	2
Signaling / communication	17
Cell structure / cytoskeleton	10
Cell defense	6
Development	10
Gene regulation	5
Metabolism	32
Protein processing	10
Protein synthesis	33
Unclassified	57

[1]182 uniquely represented ccgs were classified according to their known or predicted functions from the Broad Institute *N. crassa* Sequencing Project (http://www.genome.wi.mit.edu/annotation/fungi/neurospora/). This table includes all of the known or predicted ccgs, excluding *frq*, from Table 1-1 (20 ccgs), Correa *et al*., 2003 (140 unique ccgs), and Nowrousian *et al*., 2003 (22 unique ccgs).

rhythms in metabolism. Several genes encoding antioxidant enzymes that prevent damage due to reactive oxygen species (ROS) are also under clock control, including glutamate dehydrogenase, glutamine synthetase, oxidoreductase, and catalase. Genes encoding these enzymes peak during the daytime, suggesting that the clock increases antioxidative defense mechanisms to cope with increases in free radicals that can result from light exposure (Aguirre et al., 2005). This observation is consistent with the idea that rhythms in defense molecules play a role in the adaptive value of the circadian clock system. Rhythms in genes that encode proteins with antioxidant activity have been described in plants, chickens, mice, and humans (reviewed in Hardeland et al., 2003). Interestingly, mutations in clock genes are associated with increased oxidative damage and cancer rates, which may in turn affect life span (Hardeland et al., 2003). While ROS have been regarded as harmful byproducts of metabolism, a growing body of evidence suggests that ROS have important physiological functions (reviewed in Finkel, 2003). For instance, the redox potential of a cell can be influenced by levels of ROS through changes in conformation and oligomerization of cysteine-rich redox-sensitive proteins, including transcription factors and kinases (Adler et al., 1999; Harvey et al., 2002). This connection is particularly fascinating since changes in redox potential are known to affect the activity of the clock in bacteria and mammals. In cyanobacteria, the iron sulfur cluster protein LdpA is a component of the clock protein complex. LdpA can sense the redox state of the cell and modulate the levels of the clock component CikA to fine-tune the length of the circadian period (Ivleva et al., 2005). In mammals, the redox state of cellular NAD cofactors affects the DNA-binding affinity of heterodimers of central

clock components CLOCK:BMAL1 and NPAS2:BMAL1 *in vitro* (Rutter et al., 2001). The reduced form, NADH, promotes DNA binding of the heterodimers to the promoters of m*cry* and m*per*, and the oxidized form, NAD+, decreases it, which suggests that if the same occurs *in vivo,* the redox state of the cell may affect the phase of the core oscillator. The effects of redox potential on FRQ/WCC components in *N. crassa* have not been investigated. However, it is conceivable that WC-1 could act not only as a photoreceptor but also as a redox sensor as changes in the oxidation state of FAD bound to the LOV domain of WC-1 are thought to affect WCC transcriptional activator activity (Ballario et al., 1998; Belozerskaya, 2006). Alternatively, one of the other two PAS domains in WC-1 or the single PAS domain of WC-2 might act as a sensor of the cellular redox state (Taylor and Zhulin, 1999). Taken together, ROS, through their effect on redox potential, may function as a conserved signal to influence clock activity, and the clock in turn can regulate antioxidant levels to potentially modulate this signal.

Surprisingly, the largest group of coordinately cycling transcripts in *N. crassa* encodes ribosomal proteins, and expression of most of these genes peaks in the late night. Assuming that the ribosomal protein levels are also rhythmic (this has not been examined), these data imply that the ribosome numbers increase in the late night to prepare for times of day when most rhythmic transcripts peak. A more speculative role for rhythms in ribosomal protein levels is that changes in ribosome composition over the course of the day may affect how efficiently individual mRNAs compete for ribosomes. Different classes of mRNAs could be preferentially translated rhythmically and may

partly explain how rhythmic protein levels can arise from constitutive mRNA species, such as for *N. crassa* WC-1 (Lee et al., 2000). Several genes encoding transcription factors and proteins with known or suspected roles in signal transduction are rhythmic in *N. crassa*. Along with the 57 genes of unknown function, these are excellent candidates for components involved in signaling time-of-day information from the oscillator through the output pathways.

Finally, a new class of ccgs that cycle in expression in a strain lacking FRQ was identified from the microarray studies (Correa et al., 2003). The expression of one of these genes, *ccg-16*, peaks in the evening. *ccg-16* mRNA rhythms are generated by a temperature-responsive, temperature-compensated circadian FLO that requires functional WC-1 and WC-2 proteins for activity (de Paula et al., 2006). The FRQ/WCC oscillator and FLO may interact with each other through shared WC proteins. WC-1 and WC-2 may also function differently in the FRQ/WCC oscillator and FLO. In either case, the identification of distinct FLO-dependent ccgs provides critical tools for identifying FLO components and for understanding how a multi-oscillator system, common to eukaryotic circadian clock systems (Bell-Pedersen et al., 2005), functions to control rhythmic gene expression.

Signaling Time-of-Day Information from the Oscillator to the ccgs

It is still unclear how oscillator components signal time information through the output pathways to regulate rhythmic gene expression at different phases of the day. One

possible mechanism is direct activation by components of the FRQ/WCC oscillator. A C-box (CGAT(N)CCGCT) located in the *frq* promoter is bound by the WCC and is required for rhythmic *frq* expression (Froehlich et al., 2003a). The C-box has also been identified in the promoters of 18 ccgs (updated from Correa et al., 2003), most of which peak in the late night to early day when the WCC is most abundant in the nucleus, which suggests that these genes may be direct targets of the WCC. Four of these ccgs are known or putative transcription or signaling factors that would, in turn, be predicted to control a subset of downstream ccgs. Consistent with the idea that the WCC can directly mediate transduction of time information from the FRQ/WCC oscillator to the output pathways, 26 ccgs are rapidly induced in response to overexpression of WC-1 in dark-grown cultures (data combined from Correa et al., 2003; Lewis et al., 2002). However, only 3 of the 18 ccgs that contain a C-box are induced by overexpression of WC-1 in the dark. The ability of WC-1 to induce expression of genes that lack a C-box suggests that there may be promoter elements in addition to the C-box that can be bound by WC-1 or that effectors downstream of WC-1 activate these genes. The inability of WC-1 to induce expression of some genes that do have a C-box suggests that another transcription factor may be inhibiting the expression of these genes or may be present in limiting amounts. Seventeen of the ccgs that are induced by overexpression of WC-1 encode ribosomal proteins. The significance of this finding remains to be understood. An 8-nt element (TCTTGGCA) occurs 42 times in 29 of 69 *N. crassa* late-night-specific genes (Correa et al., 2003). This element is very similar to the core of a 45-bp fragment in the *ccg-2* promoter that is located near the start of transcription and contains a positive

activating clock element (ACE; Bell-Pedersen et al., 1996a). The ACE was shown to be both necessary and sufficient for rhythmicity, and factors are present in nuclear extracts that interact specifically with the ACE. The amount of binding and the mobility of the complexes change over the course of the day. These data suggest that the amount or activity of the factors, modification of the factors, or the addition of accessory factors is rhythmic and is consistent with these proteins having a role in clock control of the *ccg-2* gene. Experiments to identify these factors appear to rule out binding of the ACE directly by WC-1 and FRQ and suggest that activation by the clock via ACE is indirect and/or involves novel factor(s) that control the rhythmic expression of *ccg-2* (Bell-Pedersen et al., 2001; unpublished data). Lastly, many ccgs lack both ACE and the C-box, consistent with the existence of other clock control regulatory elements that may be direct targets of FRQ, regulated by other undescribed oscillator components, or indirectly regulated by known oscillator components.

In support of a hierarchical organization for the regulation of the output pathways, several ccgs encode transcription factors or signaling components that would be predicted to activate rhythmic expression of downstream genes and may be at least partially responsible for the different phases of peak ccg expression. Also consistent with a hierarchical organization, two morning-specific genes, *ccg-1* and *ccg-2*, are regulated differently by the FRQ/WCC oscillator. In standard growth media, *ccg-1* levels are constitutively high in strains that lack functional FRQ protein, whereas *ccg-2* levels are constitutively low. In strains with a normal FRQ/WCC oscillator, deletion of ACE from

the *ccg-2* promoter results in constitutive low-level expression of *ccg-2* (Bell-Pedersen et al., 1996a). The simplest model is that the FRQ/WCC oscillator regulates a repressor of *ccg-1* and an activator of *ccg-2*. However, the output pathways themselves have yet to be elucidated.

While different organisms use their clocks to regulate different biological processes at different phases during the day, several fundamental aspects of rhythmicity are conserved. Thus, we expect that elucidating the output system in *N. crassa* will help us understand the workings of the circadian clock system in more complex organisms. For example, it is clear that regulation of *N. crassa* ccgs is complex, involving multiple branched pathways and multiple oscillators. We do not yet have a complete description of an output pathway from the oscillator components to a terminal ccg in any organism. Also, there is no system where we understand how the oscillators are able to regulate the different phases of ccg expression. The identification of ccgs with potential WCC binding sites that are themselves transcription factors is one step toward this goal. The link between the circadian clock and redox/ROS is of great interest, as this connection appears to be conserved from bacteria to mammals and has the potential for an involvement in pathologies associated with a defective clock, as well as a role in aging. In *N. crassa*, circadian regulation of several genes encoding enzymes that function as antioxidants has been shown, suggesting that the clock functions to increase the ability of cells to deal with ROS during the day. In addition, we speculate that the clock can sense the cellular redox state through one of the PAS domains of WC-1 or WC-2.

Alterations of cellular redox potential by ROS levels may provide a mechanism to regulate the phase of the clock and the timing of expression of antioxidants. Studies on this linkage and the output pathway mechanisms in the versatile model organism *N. crassa* will help us further understand the central role of the clock in an organism's life.

The work presented in this dissertation represents the first identification of an output pathway from the *N. crassa* FRQ/WCC oscillator. Chapter II describes a genetic selection scheme based on the aforementioned differential expression of *ccg-1* and *ccg-2* in the absence of FRQ protein. This work was initiated by Irene March and Dr. Louis Morgan, who contributed greatly through the construction of plasmids and strains, and helped to determine the proper media conditions for the selection. Three mutant strains (COP1-2, COP1-3, and COP1-4) from this selection carry mutations that affect rhythmicity of both *ccg-1* and *ccg-2*, suggestive of a bifurcated output pathway from the FRQ/WCC oscillator. These mutations are predicted to affect components upstream of the bifurcation. Another mutant strain (COP1-1) displays altered expression of *ccg-1* mRNA but not *ccg-2*, which places that mutation downstream of the bifurcation leading to rhythmic *ccg-1* expression. This selection was the first of its kind to identify output components from any circadian system and has led to the first description of a *bona fide* circadian output pathway, which is presented in Chapter III.

In Chapter III, I describe the cloning and characterization of the gene altered in one of the above strains, COP1-4. Dr. Renato M. dePaula contributed significantly to this work

through his assistance with processing tissue and performing Western Blots. COP1-4 was found to have a mutation in the *response regulator-1* gene, which is predicted to encode a typical response regulator protein (RRG-1). RRG-1 of *N. crassa* is involved in a complex two-component signaling pathway (or phosphorelay) that functions in sensing and responding to hyperosmotic stress. The work presented here demonstrates that this pathway is co-opted by the clock in order to regulate multiple downstream effectors in a circadian manner. Additionally, circadian regulation of this pathway may allow wild-type *N. crassa* cells to anticipate hyperosmotic conditions and up-regulate the necessary genes and proteins required to protect the organism from desiccation. This study marks the first empirical evidence for the adaptive value of the circadian clock in *N. crassa* and is a major step toward elucidating the output pathways employed by the clock to regulate gene expression.

CHAPTER II

A GENETIC SELECTION FOR CIRCADIAN OUTPUT PATHWAY MUTATIONS IN *Neurospora crassa**

Introduction

Circadian (daily) rhythms are endogenous, self-sustaining oscillations that are regulated by a central pacemaker composed of one or more biochemical oscillators (Edmunds, 1988; Young and Kay, 2001). These rhythms are observed in a wide variety of organisms, ranging from daily rhythms in photosynthesis in cyanobacteria and plants and development in fungi to activity and sleep-wake cycles in rodents and humans. The core oscillators of several organisms consist of autoregulatory feedback loops, where the positive elements of the loop activate transcription of the negative elements. The negative elements then inhibit their own transcription by blocking the activity of the positive elements (Dunlap et al., 1999; Reppert and Weaver, 2002; Young and Kay, 2001).

The circadian clock system of *N. crassa* is one of the best understood (Loros and Dunlap, 2001). *N. crassa* displays an easily viewed natural 22-h rhythm of asexual

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spore development (conidiation) in constant darkness. This conidiation rhythm provided the basis for "brute force" genetic screens for mutations that affect the clock. The *frequency* (*frq*) gene was identified through such screens (Feldman and Hoyle, 1973; Loros and Feldman, 1986) and later was shown to be a core oscillator component required for circadian rhythmicity (Aronson et al., 1994a; McClung et al., 1989).

The *frq*-oscillator consists of interconnected positive and negative feedback loops involving the products of the *frq* and *white collar* (*wc-1*, *wc-2*) genes (Loros and Dunlap, 2001). The WC-1 blue light photoreceptor (Froehlich et al., 2002; He et al., 2002) forms a complex with WC-2 (White Collar Complex, WCC). This complex directly activates transcription of the *frq* gene (Froehlich et al., 2003a). Levels of FRQ protein then slowly increase. At high levels, FRQ protein binds to and inhibits the activity of the WCC (Cheng et al., 2001a; Denault et al., 2001). This causes *frq* transcription and the accumulation of FRQ protein to stop. Subsequent phosphorylation-induced decay of FRQ leads to reactivation of *frq* transcription, allowing the cycle to start again (Liu et al., 2000). In addition to the *frq*-oscillator, several lines of evidence have suggested the presence of additional oscillators in the cell (Aronson et al., 1994a; Correa et al., 2003; Lakin-Thomas and Brody, 2000; Loros and Feldman, 1986; Merrow et al., 1999; Morgan and Feldman, 2001). However, components of the other oscillator(s) have not yet been identified.

Time-of-day information is signaled from the oscillator(s) to genes and gene products residing in output pathways to generate the observed daily rhythms. Genes that are rhythmically expressed under control of the clock, and reside downstream of the output pathways from the clock, are termed *clock-controlled genes* (*ccgs*; Loros et al., 1989). To date, over 150 *ccgs* have been identified in *N. crassa* (Bell-Pedersen et al., 1996b; Correa et al., 2003; Loros et al., 1989; Nowrousian et al., 2003; Zhu et al., 2001), yet few details of the output pathways from the *Neurospora*, or any other organism's, pacemaker are known.

The functions of the *N. crassa ccg* products are varied, ranging from involvement in development to stress responses and metabolic processes. The two most highly characterized *ccg*s are the morning-specific genes *ccg-1* and *eas* (*ccg-2*; hereafter called *ccg-2* for simplicity). Clock control for both *ccg-1* and *ccg-2* requires a functional *frq*-oscillator (Arpaia et al., 1993, 1995). The abundantly expressed *ccg-2* gene encodes a member of a class of low molecular weight, cysteine-rich secreted hydrophobic proteins called hydrophobins (Bell-Pedersen et al., 1992; Lauter et al., 1992). The hydrophobins coat the outer cell wall of fungi and maintain the cell-surface hydrophobicity essential for air dispersal of mature conidiospores. Inactivation of *ccg-2* results in an "Easily Wettable" (Eas) phenotype in which the asexual conidiospores have a darkened, wetted appearance, and mix readily into water (Bell-Pedersen et al., 1992). The function of *ccg-1* is unknown; *ccg-1*-null strains do not display any discernable phenotype (Lindgren,

1994). In addition, the CCG-1 protein sequence does not share similarity to other characterized proteins, but CCG-1 is conserved among filamentous fungi.

Both the *ccg-1* and *ccg-2* genes were shown by nuclear run-on assays to be controlled by the clock, at least partially, at the level of transcription (Loros and Dunlap, 1991). Analysis of the *ccg-2* promoter localized a positive Activating Clock Element (ACE) required for *ccg-2* mRNA rhythmicity to within a 68 bp sequence located between -150 and -118 bp from the start of transcription (Bell-Pedersen et al., 1996a; Bell-Pedersen et al., 2001). Deletion of the ACE resulted in constitutive low-level expression of *ccg-2* mRNA in constant darkness, suggesting that rhythmicity results from activation of transcription by the clock. A negative promoter element required for *ccg-1* rhythmicity was identified between –444 and –91 bp upstream from the transcriptional start site (Lindgren, 1994). Deletion of this element resulted in randomly fluctuating high levels of *ccg-1* mRNA in constant darkness. These data suggested that, while *ccg-1* and *ccg-2* both peak in expression in the subjective early morning, they are regulated differently by the clock.

The use of genetics in the study of clocks has been restricted to brute force screening for altered overt rhythmicity, or misexpression of a reporter gene to identify clock-associated mutations (Golden et al., 1998; Loros and Dunlap, 2001; Millar et al., 1995). No targeted selections or suppressor screens have been described. We have utilized a genetic selection (Carattoli et al., 1995) to identify additional components of the *N.*

crassa circadian clock system by taking advantage of the clock-dependent regulation of the *ccg-1* and *ccg-2* genes.

To accomplish the selection, promoter fragments of the *ccg-1* and *ccg-2* genes containing the clock regulatory elements were fused to the *mtr* (*methyl tryptophan resistance)* gene. The *N. crassa mtr* gene encodes an amino acid permease required for uptake of neutral aliphatic and aromatic amino acids (Carattoli et al., 1995; Dillon and Stadler, 1994; Koo, 1991; Stadler et al., 1991). Loss of *mtr* function can be selected for based on resistance to the toxic amino acid analogs *p*-fluorophenylalanine (FPA) or 4-methyltryptophan (see Appendix 1). Gain of *mtr* function can be selected for on high-arginine (Arg)/ low-tryptophan (Trp) medium in a *trp-2* mutant background. Arg blocks the basic amino acid transporter, making the permease encoded by *mtr* essential for transport of Trp (see Appendix 1). The *ccg::mtr* fusions were individually transformed into a *mtr trp-2 frq*^{10} strain. The *frq*^{10} mutation is a null mutation that was produced by replacement of the *frq* open reading frame with the hygromycin resistance gene (Aronson et al., 1994a). In *frq*^{10} strains, expression of the *ccg-1::mtr* transgene is high and the cells are Mtr^{+} (grow on Trp/Arg medium, but not on FPA). Alternatively, in the *frq*^{10} strain, expression of the *ccg-2::mtr* transgene is low and the cells are Mtr^{-} (grow on FPA, but not on Trp/Arg medium). To identify genes involved in regulating *ccg-1* and *ccg-2* expression, mutations that altered the Mtr-based growth phenotype of strains containing either the *ccg-1::mtr* transgene or the *ccg-2::mtr* transgene were selected, and analyzed for expression of the endogenous *ccg-1* and *ccg-2* genes.

Materials and Methods

Plasmid construction

The -481 to +57 promoter region of the *ccg-1* gene was amplified by PCR using pKL119 (Lindgren 1994) as a template and 5'CTAGGAATTCTGTTGACCATTGGTTAC 3' and 5'CTAGAGGCCTGAGAAGAATAGAAGC 3' as primers. The -1079 to +1 promoter region of the *ccg-2* gene was amplified by PCR using pLW1K (Bell-Pedersen *et al.* 1992) as a template and the primers
5' CTAGGAATTCGTCACAGACAACGGGTGA 3' and
5' CTAGAGGCCTGGTTTGAGTGTTGTGTTG 3'. Because the basal level of *ccg-2* transcription from promoter fragments containing only the ACE is low, we used a larger promoter fragment that drives higher levels of *ccg-2* expression and contains promoter elements involved in clock (ACE) and light regulation of *ccg-2* (Bell-Pedersen et al., 1996a; Bell-Pedersen et al., 2001). However, the use of the larger *ccg-2* promoter fragment may reduce the sensitivity of the selection and allow for the isolation of mutations that affect light regulation of *ccg-2.* The primer pairs introduced an *Eco*RI site (underlined) at the 5' end and a *Stu*I site (underlined) at the 3'end of the amplified DNA. The promoters were separately cloned into pNC807 (Koo, 1991) digested with *Eco*RI and *Stu*I creating *ccg* promoter::*mtr* gene fusions. The 3.2 kb *ccg-1::mtr* and 3.6 kb *ccg-2::mtr Eco*RV fragments of the resulting plasmids were subsequently cloned into the *Eco*RV site of pMLSBml generating plasmids pCCG1M and pCCG2M, respectively. pMLSBml was kindly provided by Drs. Mari Shinohara and Jay Dunlap, Dartmouth Medical School, and contains the benomyl resistance gene isolated from pSV50 [Fungal

Genetics Stock Center, Kansas City, MO] inserted into the *Sma*I site of pBSKII+ [Stratagene, La Jolla, CA].

Strains and culture conditions

The *mtr*^{SR33} *trp-2*^{41} *mat A* strain was obtained from David Stadler (University of Washington). The *mtr* (*methyl-tryptophan resistance*) mutation carried by this strain is non-revertible due to a small deletion in the coding sequence. The *trp-2* gene encodes anthranilate synthetase (Keesey et al., 1981). The *mtr*^{SR33} *trp-2*^{41} *mat A* strain was crossed to lab strain 40-1 (*bd frq*^{10} *mat a*) to obtain strain 106-20 (*bd frq*^{10} *mtr trp-2 mat a*). The *bd* (*band*) mutation allows the circadian rhythm of conidiation to be easily observed on race tubes (Sargent et al., 1966). The *frq*^{10} null mutation was generated by replacement of the *frq* open reading frame with the hygromycin resistance gene (Aronson et al., 1994a). The mating types of the strains are indicated (*mat A* or *mat a*). The 106-20 strain was transformed with the pCCG1M or pCCG2M plasmid by electroporation and transformants were selected on benomyl-containing media using standard techniques (Chakraborty BN, 1991). The resulting CCG1M and CCG2M strains are homokaryotic isolates of the pCCG1M and pCCG2M transformants, respectively. Homokaryons were obtained from a microconidial suspension passed through a 5-micron filter (Ebbole, 1990) and germinated on medium containing benomyl. Genomic DNA isolated from the transformants was analyzed by Southern blotting to verify integration of a single plasmid into the chromosome (data not shown).

All strains used in this study were maintained on Vogel's minimal media with the appropriate supplements and handled according to standard procedures (Davis, 1970). Strains with the *frq*^{10} mutation were maintained on hygromycin B (200 μg/ml). Benomyl was added at 1 μg/ml to media before autoclaving to select for strains carrying pCCG1M or pCCG2M constructs. Strains carrying the *trp-2* mutation were supplemented with 10 μg/ml anthranilate. Anthranilate is a metabolic precursor of tryptophan that does not require *mtr* for uptake. Strains carrying the *mtr* mutation are resistant to the toxic amino acid analog *p*-fluorophenylalanine (FPA) at 60 μg/ml. Selection of mutant strains that negatively affect expression of the *ccg::mtr* transgene, resulting in low level *mtr* expression, were performed on media containing FPA, anthranilate, and benomyl (FA medium). Selection of mutant strains that positively affect expression of the transgene, resulting in high level *mtr* expression, were performed on 0.01 mg/ml Trp, 1 mg/ml Arg, and benomyl (TA medium). Race tube assays were carried out at 25°C on glucose-arginine media (0.1% glucose, 0.5% arginine, 1X-Vogel's salts, 10 μg/ml anthranilate, 2% agar). For RNA analyses, tissue was grown and synchronized by light-to-dark transfers following published procedures (Loros et al., 1989).

Mutagenesis

Conidial suspensions (15 ml) of strains CCG1M or CCG2M (10^6 conidia/ml) were placed into a sterile petri dish and exposed, with continuous shaking, to a short-wave UV light (6W) held 12 cm away. To select for mutations, dosages that corresponded to

roughly 25% and 50% survival were plated onto the appropriately supplemented media and incubated in the dark at 30°C.

Nucleic acid isolation and hybridization

RNA isolation, northern hybridization, and densitometric data analysis protocols have been described previously (Bell-Pedersen et al., 1996a). Radioactive riboprobes were synthesized from pKL119 (*ccg-1*) or pLW1K (*ccg-2*) using T3 polymerase in the presence of [α-^{32}P]UTP. Radioactive *mtr* DNA probes were generated from pNC807 using [α-^{32}P]dATP. Northern blot signals were normalized in each experiment to *rRNA*, which remains at constant levels under the growth conditions used (Loros *et al.* 1989).

Results

A genetic selection for mutations that affect expression of the clock-controlled ccg-1 *and* ccg-2 *genes*

Rhythmic expression of the morning-specific *ccg-1* and *ccg-2* genes requires *frq* (Arpaia et al., 1993, 1995); however, the expression levels of these genes in *frq*-null (*frq*^{10}) strains has not been determined. The mRNA levels of *ccg-1* and *ccg-2* were examined by northern assays from *frq*$^{+}$ and *frq*^{10} strains harvested after 12 and 20 hours of growth at 25ºC in constant darkness, representing subjective dawn (the time of peak *ccg-1* and *ccg-2* mRNA levels in wild-type strains) and subjective early evening (the time of trough *ccg-1* and *ccg-2* mRNA levels in wild-type strains), respectively (Figure 2-1A). After 20

hours in the dark (DD20), *ccg-1* mRNA levels were elevated ~10-fold in frq^{10} as compared to the frq^{+} strain. The high level of *ccg-1* mRNA at DD20 in the frq^{10} strain was similar to the peak level observed at DD12 in frq^{+}. Little difference was observed in *ccg-1* mRNA levels between the two strains at DD12. Conversely, *ccg-2* mRNA levels were ~30-times lower in frq^{10} as compared to expression in the frq^{+} strain at DD12, and the levels were low in both strains at DD20. Similar results were observed in comparisons of *ccg-1* and *ccg-2* mRNA levels between wild type and a strain that produces a truncated WC-1 protein ($wc\text{-}1^{ER53}$; Lee et al., 2000; and data not shown). These data suggest that the altered levels of *ccg-1* and *ccg-2* mRNA in the frq^{10} strain may not be a direct consequence of the absence of FRQ, but rather result from the loss of a functional *frq*-oscillator. Together, these data support the idea that *ccg-1* expression is negatively regulated and *ccg-2* expression is positively regulated by a pathway involving the *frq*-oscillator (Figure 2-1B; Bell-Pedersen et al., 1996a; Bell-Pedersen et al., 2001; Lindgren, 1994; Yang et al., 2002).

Electrophoretic mobility shift assays indicated that FRQ protein is not part of protein complexes that bind to the clock regulatory element (ACE) of *ccg-2* (Bell-Pedersen et al., 2001), supporting the hypothesis that the FRQ-mediated regulation of *ccg-2* is indirect. Thus, the gene products that link FRQ levels to expression of *ccg-2,* and the components that regulate *ccg-1* expression, are unknown. We reasoned that mutations in genes in the circadian output pathways could be identified by finding mutant strains that

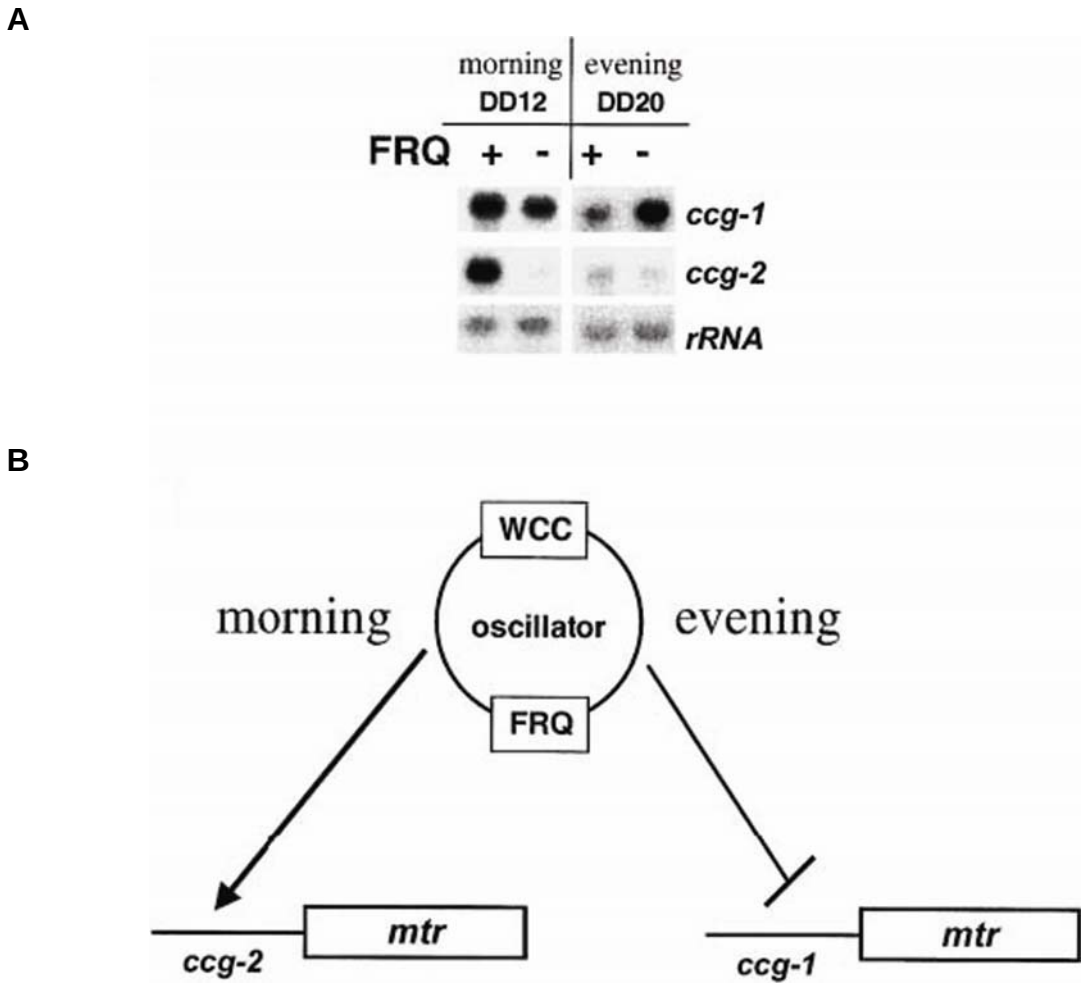


Figure 2-1. The clock-controlled *ccg-1* and *ccg-2* genes are differentially regulated by the clock. (A) The levels of *ccg-1* and *ccg-2* mRNA were assayed by northern blot analyses in the frq+ strain 87-3 and the frq10 strain 40-1. Liquid cultures of mycelia were grown in constant darkness and harvested after 12 (DD12) and 20 (DD20) hours in the dark, representing subjective morning and early evening, respectively. Equivalent loading of RNA was verified by hybridization to rRNA. (B) A simple model representing the *N. crassa* frq-oscillator with output pathways from the clock positively regulating rhythmic levels of *ccg-2* mRNA in the morning and negatively regulating rhythmic levels of *ccg-1* in the evening (see the text for details).

express low levels of *ccg-1* mRNA or high levels of *ccg-2* mRNA in the *frq*^{10} background.

Promoter regions containing sequences required for circadian expression of *ccg-1* and *ccg-2* were independently fused to the coding sequence of the *mtr* gene to produce pCCG1M and pCCG2M, respectively (Materials and Methods). The benomyl-resistance pCCG1M and pCCG2M plasmids were transformed into 106-20 (*bd mtr trp-2 frq*^{10}) to produce the CCG1M and CCG2M strains, respectively. These transformants were chosen based on their growth phenotypes in liquid culture at 25ºC (Table 2-1). Control strain 106-20 did not grow in high Arg/low Trp (TA) media, but did grow in FPA/anthranilate (FA) media reflecting the Mtr^{-} phenotype and inability of this strain to uptake Trp. Strain CCG1M grew in TA, but not in FA media. This growth pattern suggested high expression of the *ccg-1::mtr* fusion in the *frq*^{10} strain and the ability of this strain to import both Trp and FPA. Strain CCG2M grew well in FA and had very little growth in TA media. This growth pattern suggested a low level of expression of the *ccg-2::mtr* fusion and decreased ability of this strain to import both Trp and FPA.

To establish conditions for the selection of mutations, strains CCG1M and CCG2M were examined on TA and FA plating media following two days of growth in the dark at 30°C (Figure 2-2). Optimal conditions were found with plating media containing Trp at 0.01 mg/ml plus 1 mg/ml Arg or 60 µg/ml FPA. Under these conditions, the CCG1M strain grew on TA but not on FA; the CCG2M strain grew on FA, but not on TA.

Isolation of mutant strains that affect expression of ccg-1 *and* ccg-2

UV mutagenesis was conducted on strain CCG1M to obtain mutant strains that grew on plating medium containing FA and on strain CCG2M to obtain mutant strains that grew on TA medium. Eighty mutant strains were obtained from the CCG1M strain and 165 mutant strains from the CCG2M strain out of ~$6X10^5$ and $2X10^5$ cells, respectively. The mutant strains were examined for growth on minimal, FA, and TA media. Some mutant strains (5/165) selected from CCG2M that grew on TA medium were also able to grow on minimal medium. These data suggested that these strains no longer required Trp for growth, and, therefore, were undesired *trp-2* revertants or supressors (data not shown). These strains were not studied further. In addition, most (152) of the 160 CCG2M mutants which showed some growth on TA medium grew poorly on this medium and/or were still able to grow on FA medium (data not shown). Of the remaining eight FPA-sensitive CCG2M mutant strains, we examined the levels of endogenous *ccg-2* mRNA in cultures harvested after 20 hours in the dark (DD20). This time of harvest represents subjective early evening, a time of day when cycling *ccg-2* levels are normally low in wild-type strains (Loros et al., 1989) and in the frq^{10} strain (Figure 2-1). We reasoned that mutations that affect clock regulation of *ccg-2* should result in increased levels of *ccg-2* mRNA in the mutant strains as compared to the CCG2M strain. Indeed, the eight FPA-sensitive mutant strains, designated as COP2-1 through COP2-8 (circadian output pathway derived from *ccg-2*), showed increased levels of endogenous *ccg-2* mRNA as compared to the CCG2M strain (Figure 2-3A). The eight COP2 strains developed normally; however, because they carried the frq^{10} mutation, the conidiation rhythm could

Table 2-1
Strains used in this study

Strain[a]	Genotype[b]	TA	FA	Phenotype	Other comments
30-7	*bd mat A*	+	−	wt	22.6-hr period
87-3	*bd mat a*	+	−	wt	
40-1	*bd frq*^{10} *mat a*	+	−	Pale orange conidia	
181	*mtr*^{SR33} *trp-2*^{41} *mat a*	−	+	wt	
96-11	*mtr bd trp-2 mat A*	−	+	wt	
106-20	*mtr bd trp-2 frq*^{10} *mat a*	−	+	Pale orange conidia	
CCG1M	106-20 *ccg-1::mtr*(EC)	+	−	Pale orange conidia	
CCG2M	106-20 *ccg-2::mtr*(EC)	−	+	Pale orange conidia	
COP1-1	106-20 *ccg-1::mtr* (EC) *cop1-1*	−	+	Pale orange conidia	
COP1-2	106-20 *ccg-1::mtr*(EC) *cop1-2*	−	+	Eas-like, odor	Recessive
COP1-3	106-20 *ccg-1::mtr*(EC) *cop1-3*	−	+	Eas-like, odor	Recessive
COP1-4	106-20 *ccg-1::mtr*(EC) *cop1-4*	−	+	Eas-like, odor	Recessive
COP1-5	106-20 *ccg-1::mtr*(EC) *cop1-5*	−	+	Eas-like, strong odor, sterile	Dominant
COP1-6	106-20 *ccg-1::mtr*(EC) *cop1-6*	−	+	Eas-like, strong odor, sterile	Dominant
COP1-2F+	*bd cop1-2*	+	−	Eas-like, odor	Arrhythmic
COP1-3F+	*bd cop1-3*	+	−	Eas-like, odor	20.1-hr period
COP1-4F+	*bd cop1-4*	+	−	Eas-like, odor	19.9-hr period

wt, wild type.
[a] F+ refers to the strains carrying the wild-type *frq* gene.
[b] EC, ectopic integration of plasmid.

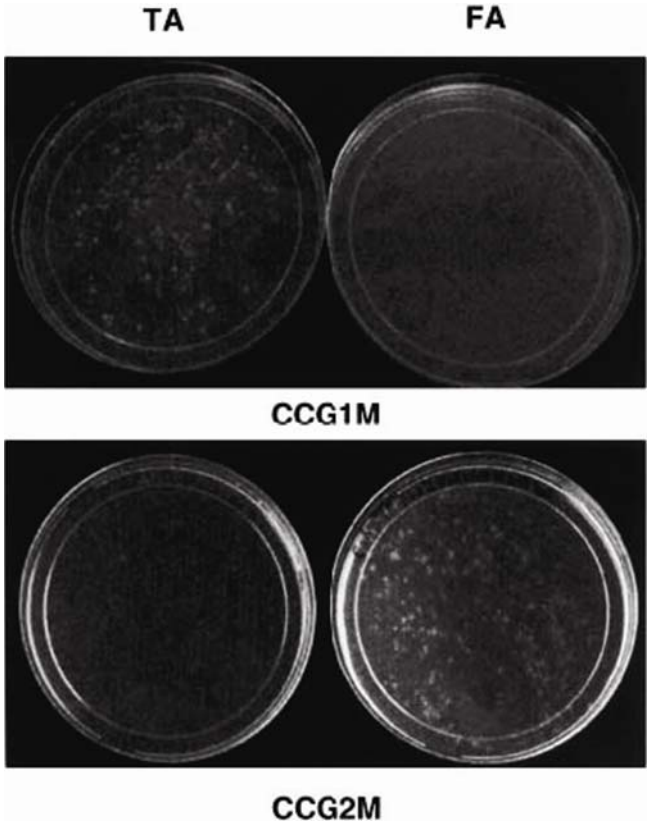


Figure 2-2. Reconstruction experiments to demonstrate the selection scheme for mutations in clock output pathways. Strains CCG1M and CCG2M were spread onto plating medium containing tryptophan/arginine (TA) or p-fluorophenylalanine/anthranilate (FA) and photographed after two days of growth in the dark at 30°C.

not be observed using standard race tube assays (data not shown). Thus, other than the Mtr^{+} phenotype, these strains lacked any readily observable phenotype that could be followed in standard genetic crosses. More importantly, the eight COP2 strains were sterile in crosses (data not shown). This phenotype might suggest that the mutations affect several ccgs, including those involved in mating (Bobrowicz et al., 2002). Because we were unable to readily cross the COP2 strains to isolate a single mutation associated with altered expression of *ccg-2*, these strains have not yet been further characterized.

Twenty-two of the 80 mutant strains isolated from the CCG1M strain grew poorly on selective FA medium, or also grew on TA medium. The other 58 FPA-resistant mutant strains derived from the CCG1M strain, designated as COP1-1 through COP1-58 (circadian output pathway derived from *ccg-1*), were examined by northern assays for endogenous *ccg-1* mRNA levels from cultures that were harvested after 20 hours of growth in the dark (DD20). This represents a time of day when cycling *ccg-1* levels are normally low in wild-type strains (Loros et al., 1989) and high in frq^{10} strains (Figure 1). A representative northern blot is shown in Figure 2-3B. The amounts of *ccg-1* mRNA from some of the 58 FPA-resistant COP1 strains were reduced from <10-times to slightly less than the amount of *ccg-1* mRNA detected in the unmutagenized CCG1M strain. Eighteen FPA-resistant COP1 strains had endogenous *ccg-1* mRNA levels that were equal to, slightly less than, or slightly greater than that observed in the control *bd*

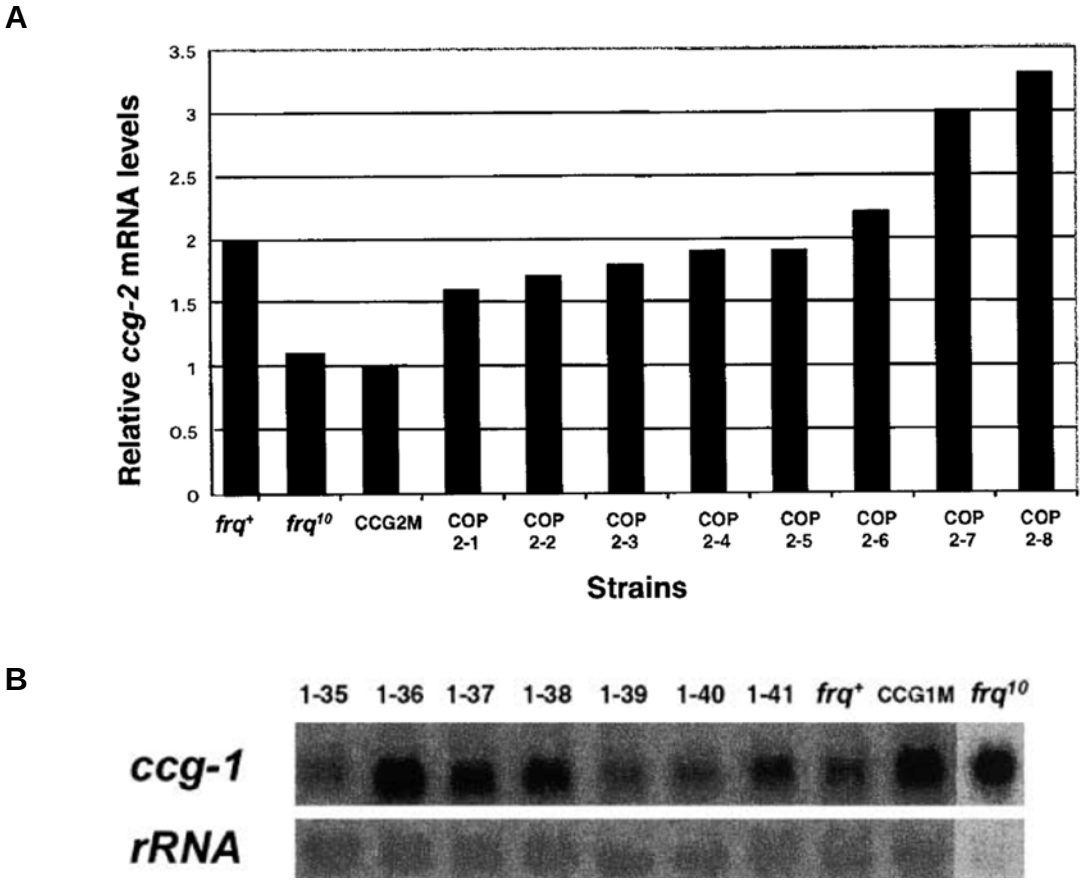


Figure 2-3. Endogenous *ccg-1* and *ccg-2* mRNA levels in selected mutant strains. (A) Densitometry of *ccg-2* mRNA levels in control strains (*bd frq*$^{+}$ strain 30-7, *bd frq*^{10} strain 40-1 and the CCG2M strain) and COP2-1 through COP2-8 mutant strains. The strains are indicated on the X-axis and the relative levels of *ccg-2* mRNA are indicated on the Y-axis. (B) A representative northern blot showing *ccg-1* mRNA levels from control (30-7, 40-1, and CCG1M) and seven COP1 mutant strains (1-35 to 1-41). rRNA is shown as a loading control.

C

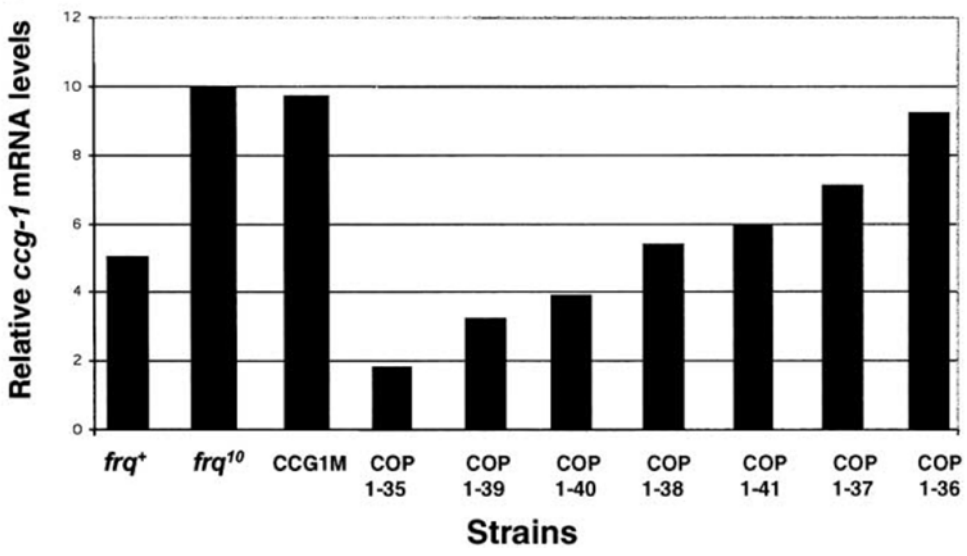


Figure 2-3 Continued.

(C) Densitometry of *ccg-1* mRNA levels in control (30-7, 40-1 and CCG1M) and COP1 mutant strains derived from CCG1M. The strains are indicated on the X-axis and the relative levels of *ccg-1* mRNA are indicated on the Y-axis. In each experiment, RNA was isolated from cultures harvested following 20 hours of growth in the dark, representing subjective early evening.

frq^{+} strain 30-7. We reasoned that mutations that reduce *ccg-1* mRNA levels in the *frq*^{10} strain to near the *frq*$^{+}$ strain at DD20, the low point in the cycle, would likely represent mutations that affect clock-control of *ccg-1*. Thus, these eighteen FPA-resistant COP1 mutant strains were chosen for further study.

Characterization of the COP1 mutant strains

We focused on 18 COP1 strains that grew on FA, but not TA, media and exhibited *ccg-1* expression levels that were comparable to the mRNA levels observed in the *bd frq*$^{+}$ strain 30-7 at DD20. Two major groups of phenotypes were observed among homokaryotic isolates of these strains. One group (n=13) was visually similar to the parental CCG1M strain (data not shown). A second group (n=5) showed an Eas-like phenotype of wetted conidia on slants, and the conidia were more readily suspended in water than the unmutagenized CCG1M strain (Table 2-1 and Figure 2-4). The five COP1 strains with the Eas-like phenotype (COP1-2 through COP1-6) also showed the increased pigmentation phenotype associated with the *eas* mutation. For each of the five Eas-like COP1 mutant strains, the phenotype was slightly less severe than the Eas phenotype. In addition, each of the five Eas-like COP1 strains had an unpleasant odor (Table 2-1). This phenotype was heritable; progeny from crosses with three out of three of the COP1 strains (COP1-2, COP1-3, and COP1-4) also had the odor associated with the Eas-like phenotype (data not shown, see Table 2-1).

The COP1-2 through COP1-6 strains were of special interest because the Eas phenotype

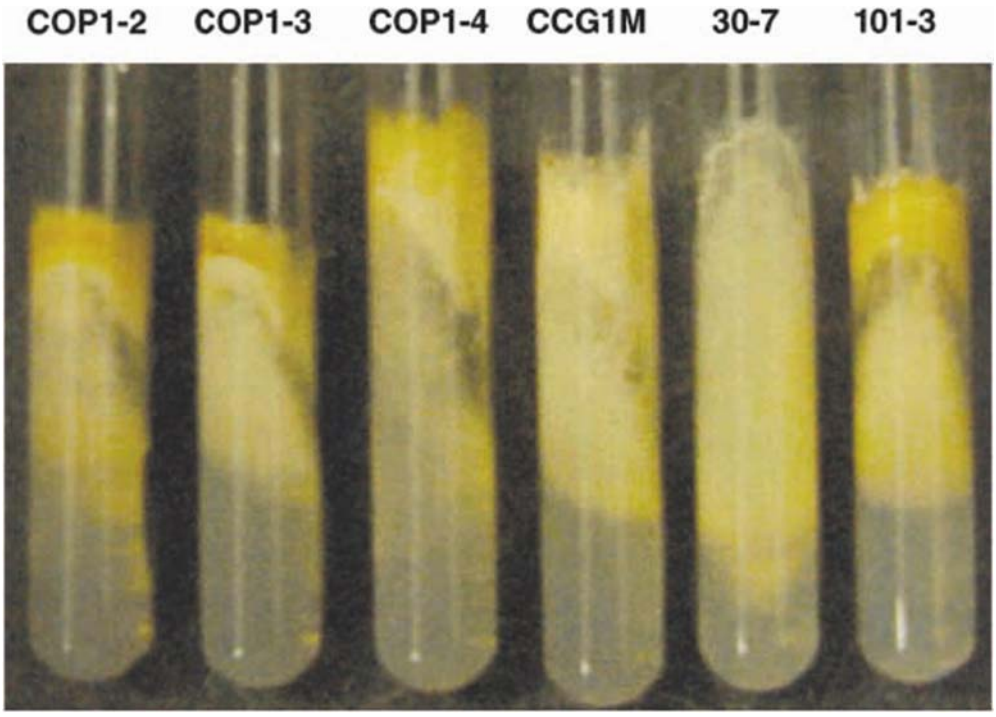


Figure 2-4. Several COP1 mutant strains selected from the CCG1M strain have an Eas-like phenotype. Slant cultures for control strain CCG1M, *bd frq*$^{+}$ strain 30-7, and 101-3 (*bd eas*^{UCLA191} *mat A*) along with homokaryotic isolates of three representative Eas-like mutant strains (COP1-2, COP1-3, and COP1-4) are shown. The Eas phenotype is characterized by dark, wetted asexual conidiospores.

is caused by loss of function of the *ccg-2* gene (Bell-Pedersen et al., 1992; Lauter et al., 1992), but the mutations were isolated from the *ccg-1::mtr* selection. These data suggested the possibility that a single mutation affected expression of not only *ccg-1*, but also of other *ccgs*. To test this hypothesis, the five Eas-like COP1 mutant strains were first examined for endogenous *ccg-1* and *ccg-2* mRNA levels, along with expression of the *mtr* transgene from cultures grown in the dark and harvested after 20 hours (Figure 2-5). As previously observed, in each mutant strain, the levels of endogenous *ccg-1* mRNA and *mtr* mRNA from the transgene were lower than the control unmutagenized strain. Furthermore, the levels of endogenous *ccg-2* transcripts were reduced in each of the five Eas-like COP1 mutant strains from their already low levels in the CCG1M parental strain. However, there was notable variability in the levels of *ccg-2* transcripts, ranging from slightly lower in COP1-5 to almost undetectable in COP1-3. These data suggest that different loci, or the severity of mutation of a single locus, resulted in different effects on *ccg-1* and *ccg-2* expression levels. None of the five Eas-like COP1 mutant strains displayed the characteristic *wc-1* or *wc-2* mutant phenotype of white hyphae resulting from reduced carotenoids (Ballario et al., 1998) indicating that the mutations were not in either of these genes, or in genes that greatly alter WC-1 or WC-2 function.

We also examined *ccg-2* mRNA levels in the other 13 FPA-resistant mutants from CCG1M that exhibited reduced *ccg-1* expression, but did not display the Eas

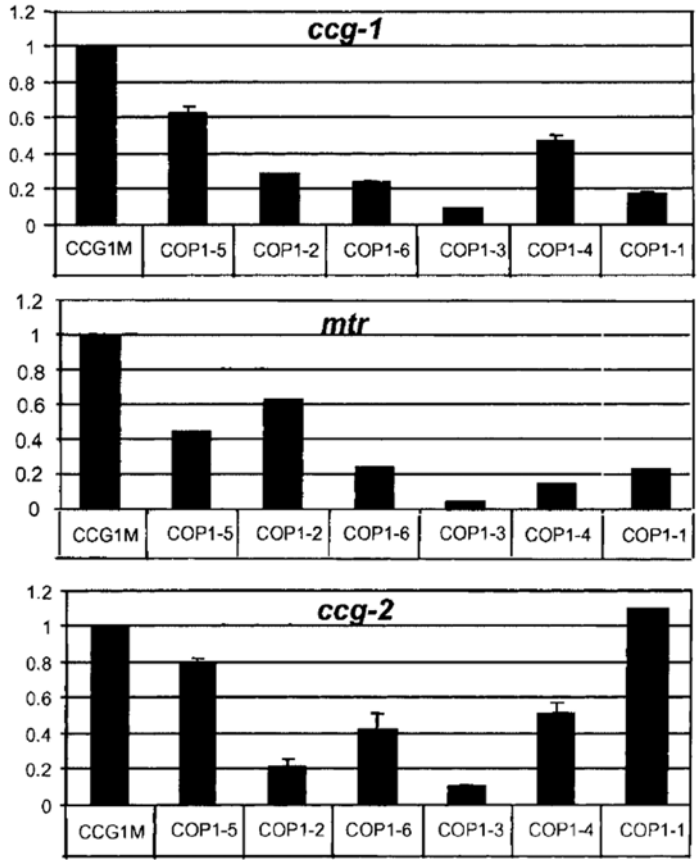


Figure 2-5. The Eas-like COP1 mutant strains have reduced levels of *ccg-2* mRNA. Densitometry of *ccg-1*, *mtr*, and *ccg-2* mRNA levels from northern blots (not shown) in the control CCG1M strain and COP1-2, COP1-3, and COP1-4 mutant strains. In each experiment, the levels of mRNA from the CCG1M strain were normalized to 1. The strains are indicated on the X-axis and the relative levels of mRNA are indicated on the Y-axis. Four independent experiments were used to determine the standard deviation of RNA levels for *ccg-1* and *ccg-2*. In each experiment, RNA was isolated from cultures harvested following 20 hours of growth in the dark, representing subjective early evening.

phenotype. One representative mutant strain, COP1-1, is shown in Figure 2-5. For COP1-1, the levels of *ccg-2* mRNA were similar to the levels observed in the CCG1M strain, suggesting that the defect in the COP1-1 mutant strain specifically affects *ccg-1* gene regulation.

To determine if the effects on *ccg-1* and *ccg-2* gene expression in the COP1-2 through COP1-6 strains were the result of single mutations, each strain was crossed to the *bd* frq^{+} strain 30-7. Haploid progeny were scored for the Eas-like phenotype and for low level endogenous *ccg-1* expression at DD20. Both frq^{+} and frq^{10} progeny with low-level endogenous *ccg-1* expression and the Eas-like phenotype were obtained from the COP1-2, COP1-3, and COP1-4 strains. In all first- and second-generation crosses, the Eas-like phenotype segregated 100% of the time with low-level *ccg-1* mRNA ($n \geq 9$; Figure 2-6 and data not shown). These data suggested that the mutant phenotypes in the COP1-2, COP1-3, and COP1-4 strains are due to mutation of a single gene. The COP1-5 and COP1-6 strains were infertile, and therefore were not examined further here.

We used forced heterokaryon analyses, in which different haploid nuclei are present in the same cell, to determine if the mutations in the five Eas-like COP1 strains are dominant or recessive to the wild-type allele (Davis, 1970). The mutations in the COP1-5 and COP1-6 strains showed dominance to the wild-type allele for the Eas-like phenotype, whereas the mutations in the COP1-2, COP1-3 and COP1-4 strains were recessive (data not shown).

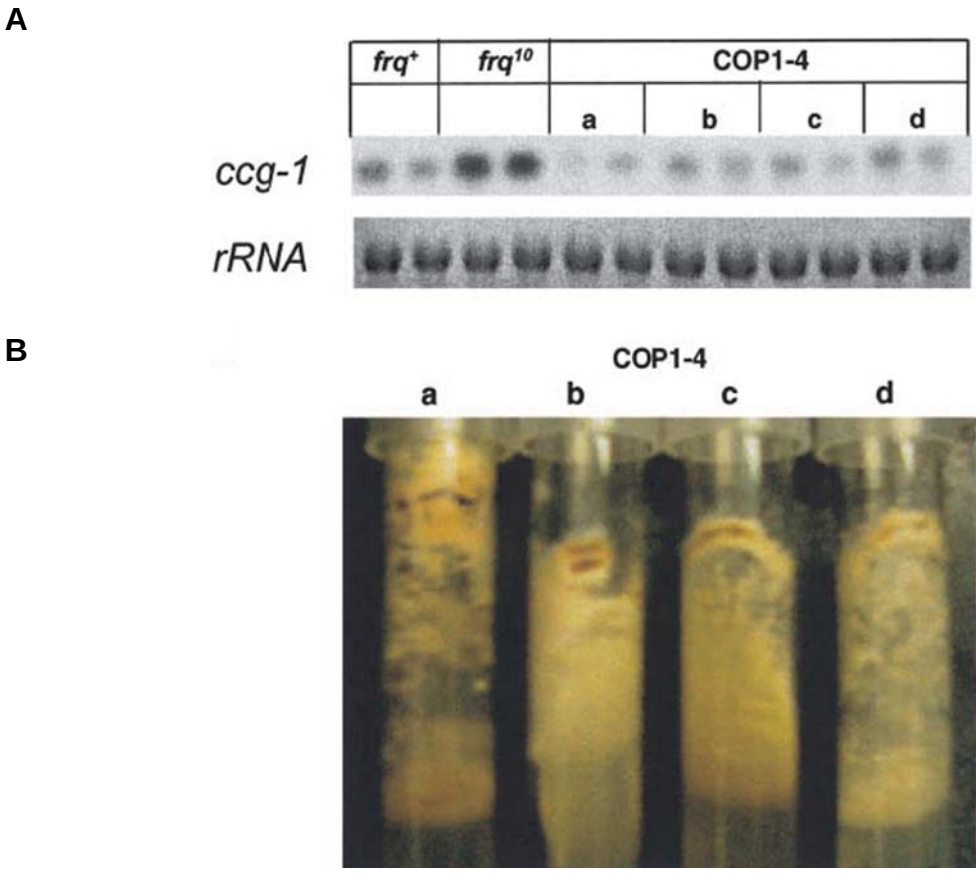


Figure 2-6. The mutant phenotypes in the COP1-4 strain are due to mutation of a single gene. (A) Progeny from a second generation cross of the COP1-4 strain were examined for *ccg-1* mRNA levels after 20 hours of growth in the dark (DD20). RNA isolated from duplicate cultures of control *bd* frq^{+} strain 30-7 and *bd* frq^{10} strain 40-1, and four frq^{10} carrying progeny of COP1-4 (labeled a, b, c and d) was hybridized to a *ccg-1* probe. Ethidium bromide stained rRNA is shown below to demonstrate equal loading of the gel. (B) Slant cultures of the COP1-4 progeny strains a-d are shown to illustrate the Eas-like phenotype.

The mutations in the COP1-1, COP1-2, and COP1-4 strains affect overt rhythmicity and circadian clock output

To examine if the *frq*-oscillator is affected in the COP1-2, COP1-3, and COP1-4 mutant strains we examined progeny from crosses with the 30-7 strain (*bd* frq^{+}). Race tube assays were used to examine the conidiation rhythms of frq^{+} COP1-2, COP1-3, and COP1-4 mutant strains (designated as COP1-2F+, COP1-3F+, COP1-4F+ strains; Table 2-1), along with the parental frq^{10} mutant strains (Figure 2-7A). Of the frq^{+} progeny, the COP1-3F+ and COP1-4F+ strains displayed conidiation rhythms with periods of 20.1 ± 0.1 h and 19.9 ± 0.4 h, respectively. This ~20-h rhythm observed in both mutant strains is about two hours shorter than observed in the wild-type clock strain 30-7. Following transition from light to dark, the COP1-2F+ mutant strain was rhythmic for 2-4 days, but produced qualitatively low levels of conidia. After this initial period, the COP1-2F+ strain became arrhythmic and produced more conidia. These data are consistent with the mutations in the COP1-2, COP1-3, and COP1-4 strains affecting either the conidiation output pathway or the circadian oscillator itself. Interestingly, the parental COP1-2 strain (which carries the frq^{10} allele) displayed rhythmic conidiation in constant darkness in about one out of 10 experiments, whereas COP1-3 and COP1-4 strains were always arrhythmic. Representative race tubes of the COP1-2 strain are shown in Figure 2-7B. Diagnostic PCR was used to confirm that these COP1-2 cells harbored the frq^{10} allele (data not shown). Because the COP1-2 strain is capable of rhythmicity in the frq^{10} background, albeit erratic, these data suggest that the mutation present in the COP1-2 strain is a partial suppressor of the *frq*-null phenotype.

A

Figure 2-7. Conidiation rhythms are altered in the Eas-like COP1 mutant strains. (A) Race tube assays show the conidiation rhythms of control *bd frq*$^{+}$ strain 30-7 and CCG1M strains, along with mutant strains COP1-2, COP1-3, and COP1-4. Progeny from each of these strains that carry the *frq*$^{+}$ allele are also shown (labeled F+). Two representative race tubes are shown for the COP1-2F+ strain. Race tubes were incubated in the light for one day at 25ºC and then transferred to the dark (25ºC). The solid lines indicate the growth front after 24 hr. The period lengths of the rhythmic strains are indicated on the left ($n \geq 18$; standard deviations are indicated).

B

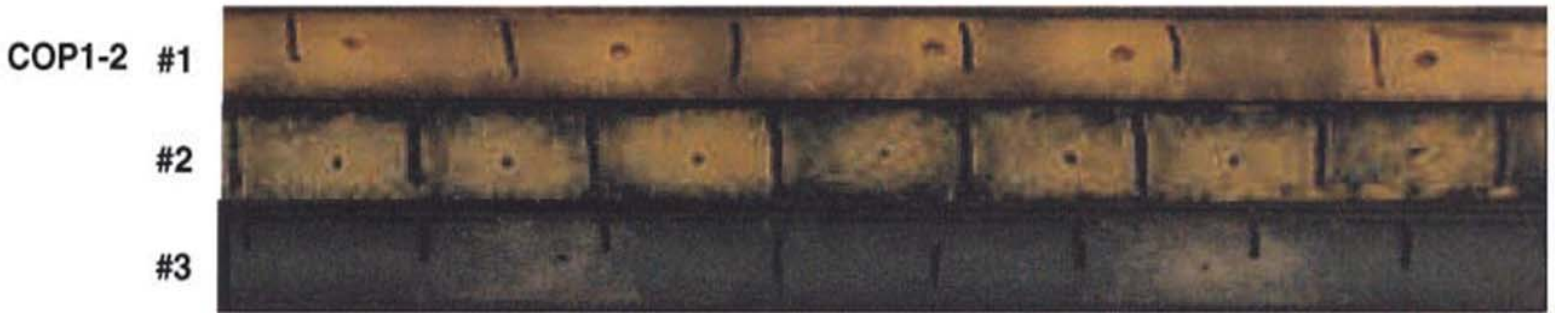


Figure 2-7 Continued.

(B) Race tube assay of the COP1-2 strains in the *frq*^{10} background. Several independent experiments are shown to depict the unusual rhythm in conidiation for COP1-2 strains that lack *frq*.

The COP1-2F+, COP1-3F+, and COP1-4F+ progeny strains were also examined by northern assays for circadian rhythms in *ccg-1* and *ccg-2* mRNA accumulation (Figure 2-8A & 2-8B). In the control 30-7 (*bd frq*$^{+}$*)* strain, both *ccg-1* and *ccg-2* mRNA accumulated rhythmically and peaked in mRNA levels in the subjective morning (8-16 hours in the dark on day 1, and 36-40 hours in the dark on day 2). Both COP1-2F+ and COP1-3F+ strains had randomly fluctuating, but primarily low, levels of *ccg-1* and *ccg-2* mRNA over the course of the day. Interestingly, the COP1-4F+ strain had widely variable levels of both *ccg-1* and *ccg-2* mRNA. However, *ccg-1* or *ccg-2* mRNA levels in the COP1-4F+ strain did not display a typical circadian pattern. These data demonstrate that circadian regulation of *ccg-1* and *ccg-2* is defective in the COP1-2F+, COP1-3F+, and COP1-4F+ mutant strains.

Discussion

The output pathway from the clock appears to involve a repressor of ccg-1 *and an activator of* ccg-2

In this study, we utilized a selection for mutations (Carattoli et al., 1995) that affect the expression of two clock-controlled genes, *ccg-1* and *ccg-2,* based on the differential expression of the *ccgs* in response to the absence of the clock component FRQ. Analysis of the *ccg-1* promoter suggests that clock regulation of *ccg-1* involves a repressor (Lindgren, 1994). Conversely, deletion of the *ccg-2* ACE results in low level arrhythmic expression, indicating that an activator of transcription is involved

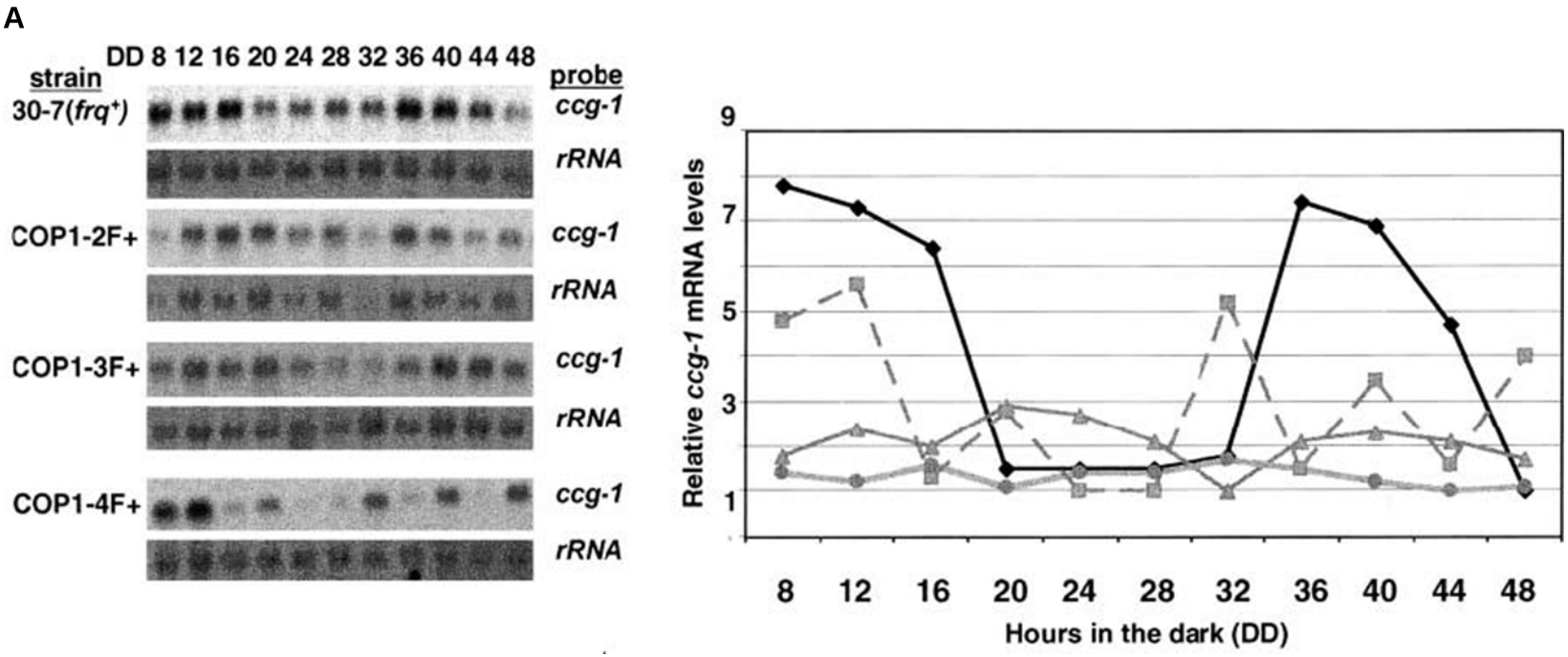


Figure 2-8. The *ccg-1* and *ccg-2* mRNA levels are arrhythmic in the Eas-like COP1 mutant strains. The levels of *ccg-1* and *ccg-2* mRNA were assayed by northern blots in control *bd frq*$^{+}$ strain 30-7 and the COP1-2, COP1-3 and COP1-4 mutant strains in the *frq*$^{+}$ background (labeled COP1-2F+, COP1-3F+, and COP1-4F+). Liquid cultures of mycelia were grown in constant darkness and harvested after the indicated times in the dark (DD). The RNA was hybridized with either *ccg-1* (A) or *ccg-2* (B) probes and rRNA probes for normalization. Following autoradiography, mRNA levels were normalized to rRNA, which remains at constant levels under these conditions (LOROS *et al*. 1989), and plotted as relative band intensity versus time in the dark. The lowest point for each individual blot was set to 1. For both A and B plots, solid black lines with filled diamonds represent mRNA from 30-7; light gray lines with filled circles represent COP1-2F+; gray solid lines with filled triangles represent COP1-3F+; and dashed gray lines with filled squares represent COP1-4F+.

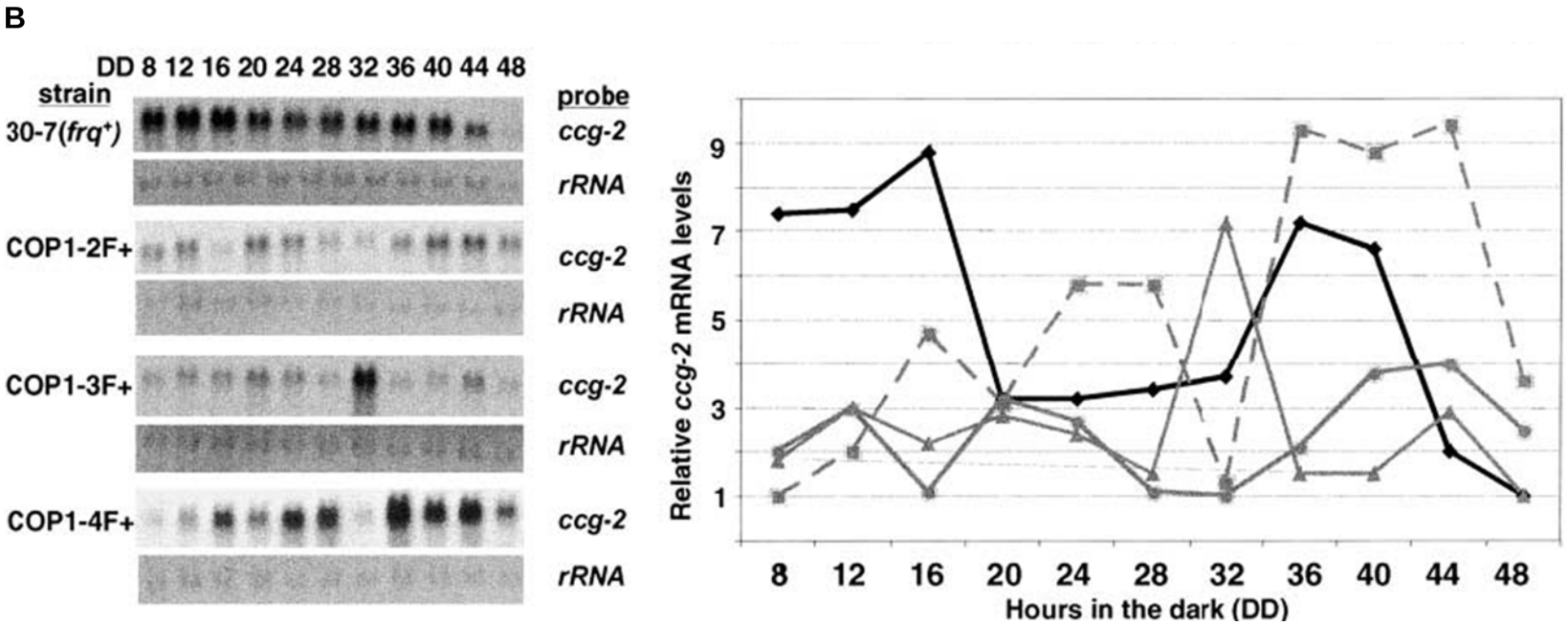


Figure 2-8 Continued.

in rhythmic transcription of *ccg-2* (Bell-Pedersen et al., 2001). One simple model to explain this regulation would be that the *frq* oscillator regulates two independent pathways: one that induces a repressor of *ccg-1* in the evening and another that induces an activator of *ccg-2* in the morning (Figure 2-1B). In the absence of the *frq*-oscillator, the repressor of *ccg-1* and the activator of *ccg-2* would both have reduced activity, resulting in high levels of *ccg-1* mRNA and low levels of *ccg-2* mRNA at all times of day. Based on this simple model, we anticipated identifying mutations that affected just *ccg-1* or just *ccg-2* expression levels. Some of the identified mutations in the frq^{10} background indeed caused reduced *ccg-1* mRNA levels, but had no effect on the levels of *ccg-2* mRNA (*e.g.* the mutation in the COP1-1 strain, Figure 2-5).

Identification of single gene mutations that affect both ccg-1 *and* ccg-2 *gene expression*

The two COP1 mutant strains that had reduced expression of both *ccg-1* and *ccg-2* grew and developed normal conidiospores (with the exception of the Eas-like phenotype), suggesting that the mutations did not affect the general transcriptional machinery or primary development. Importantly, the Eas-like phenotype and low-level *ccg-1* expression in the frq^{10} background segregated together in crosses, implying that the phenotypes are the result of a single mutation. Furthermore, previous studies have shown that the overall expression levels and rhythmicity of *ccg-2* mRNA are unaffected in strains that lack *ccg-1* (Lindgren, 1994), and that the overall expression levels and rhythmicity of *ccg-1* mRNA are unaffected in strains that have low level constitutive *ccg-2* mRNA expression (Bell-Pedersen et al., 1996a). Together, these data suggest that

the simple model of *frq*-oscillator-regulation of two distinct output pathways arising directly from the oscillator, one pathway involving a *ccg-1* repressor and a second pathway involving an activator of *ccg-2* (Figure 2-1B), is not correct.

One mechanism to explain how a single mutation could cause the reduction of expression for both *ccg-1* and *ccg-2* mRNA in the frq^{10} background is that the mutation simultaneously increases the activity of a repressor of *ccg-1* and decreases activity of an activator of *ccg-2*. In this scenario, a bifurcation in the clock output pathway from the *frq*-oscillator to the *ccg-1* and *ccg-2* genes would be predicted. In the frq^{+} background, the mutations present in the COP1-2F+, COP1-3F+, and COP1-4F+ strains abolish *ccg-1* and *ccg-2* rhythmicity. The gene(s) mutated in these strains could function in an output pathway upstream of the bifurcation, or may possibly act in the *frq*-oscillator or in a second coupled oscillator. However, the lack of a correlation between the race tube data, in which period defects in the conidiation rhythm are observed in the COP1-3 and COP1-4 strains, and the complete lack of rhythmicity of *ccg-1* and *ccg-2* mRNA levels in the same strains suggests that the mutations directly affect the output pathways rather than the oscillator itself. The mutation in the COP1-1 strain, on the other hand, appears to be specific for *ccg-1* regulation, and thus the gene product likely functions downstream of the branch point. Verification of these hypotheses will await a molecular examination of oscillator components in the COP1-1, COP1-2, COP1-3, and COP1-4 mutant strains and the cloning and analyses of the genes uncovered by the mutations.

One of the most interesting mutant strains studied was COP1-2, because in the *frq*^{10} background this strain often showed unusual and inconsistent rhythms in conidial development on race tubes. The reason for the variability in observing the rhythm and of the rhythm itself is not known at this time, but may be related to the nature of the mutation and the growth conditions used to examine the rhythms. When the COP1-2 strain was crossed to isolate the mutation in the *frq*$^{+}$ background (COP1-2F+), haploid progeny that were Eas-like also showed unusual rhythms; the strains were rhythmic for up to four days and then became arrhythmic. Interestingly, rhythmicity corresponded to low levels of conidiospore production, whereas arrhythmicity correlated with higher levels of conidiospores. Furthermore, the rhythm observed in the parental COP1-2 strain (carrying the *frq*^{10} allele) differs substantially from the overt conidiation rhythms that are sometimes observed in *frq*-null strains (Aronson et al., 1994a; Loros and Feldman, 1986). These *frq*-null rhythms have been attributed to a second cellular oscillator called the FRQ-less oscillator (FLO; Loros and Dunlap, 2001; Merrow et al., 1999). However, with the exception of COP1-2 strains, strains carrying the *frq*^{10} allele were arrhythmic in our growth conditions. Thus, it is possible that the erratic rhythm observed in the COP1-2 strain is due to its effect on the FLO, which results in partial suppression of the *frq*-null phenotype.

Each of the five Eas-like COP1 mutant strains had an unpleasant odor. The volatile molecule responsible for the smell is not known. During mating, strains of each mating type secrete a mating-type-specific pheromone that gives off a strong odor similar to the

odor given off by the five Eas-like Cop1 mutant strains. However, because the pheromones have not been purified, we do not know if the odor from the Eas-like Cop1 mutant strains is the pheromone. The *N. crassa* pheromone genes *ccg-4* (*mfA*) and *mfa* are regulated by the circadian clock (Bobrowicz et al., 2002). Therefore, it is possible that the effect on the output pathways in the Eas-like COP1 mutant strains also causes misexpression of the pheromone genes and that this is responsible for the odor. Assuming that high levels of pheromone have an effect on fertility, this idea is consistent with the finding that while the COP1-2, COP1-3, and COP1-4 strains were fertile in crosses, the level of fertility was reduced. The two strains with the strongest odor, COP1-5 and COP1-6, were infertile in crosses. Alternatively, the reduced fertility of the mutant strains could be related to the benomyl resistance gene that is present in the transformed strains (Staben, 1989).

The wild-type genes specified by the recessive mutations in the FPA-resistant COP1-2, COP1-3, and COP1-4 strains can now be cloned through complementation with existing cosmid or genomic libraries to identify clones that complement the loss of *mtr* function (*i.e.* growth on TA), but that do not themselves contain *mtr*. Furthermore, this selection scheme is now being used to identify mutations that affect expression of *ccg-1* and *ccg-2* in the frq^{+} background, and will be used in the future to select for extragenic suppressors of existing mutations using the opposite selection.

CHAPTER III

A CIRCADIAN OUTPUT PATHWAY INVOLVING RESPONSE REGULATOR-1 OF *Neurospora crassa*

Introduction

Endogenous circadian clocks provide an adaptive advantage to organisms by permitting the anticipation of, and preparation for, predictable daily rhythms that occur as a result of the Earth's rotation about its axis (Dodd et al., 2005; Ouyang et al., 1998; Sharma, 2003). Circadian clocks regulate daily rhythms in biological processes at every level, ranging from gene expression in single cells, enzyme and hormone production in organs and glands, to the sleep/wake cycles of an entire organism (Bell-Pedersen et al., 2005; Schibler, 2006). The resultant rhythms that occur at each of these levels are synchronized to the environmental cycle such that the appropriate responses are mounted at the appropriate time of day (Bell-Pedersen, 2000)

The circadian clock system of *N. crassa crassa* has been studied extensively for over 40 years and is one of the best-understood circadian models (Loros and Dunlap, 2001). *N. crassa* displays an easily observable 22-hour rhythm in asexual spore development (conidiation; Pittendrigh et al., 1959), as well as rhythms in gene expression (Loros et al., 1989), metabolism (Shinohara et al., 1998), pheromone production (Bobrowicz et al., 2002), stress response (Shinohara et al., 2002), and other processes (for review see Vitalini et al., 2006). Due to the ease with which the conidiation rhythm can be

monitored, it has been studied the most extensively. Early 'brute force' genetic screens for mutations that affect this rhythm led to the identification of the *frequency* (*frq*) gene (Feldman and Hoyle, 1973), which was later found to be a component of the core FRQ/WCC circadian oscillator (Aronson et al., 1994a; Aronson et al., 1994b; McClung et al., 1989).

The FRQ/WCC oscillator, necessary for many of the observed rhythms in *N. crassa*, is the result of an autoregulatory, transcriptional/translational feedback loop involving the *frq* and *white collar* (*wc-1, wc-2*) genes and their protein products (Loros and Dunlap, 2001). The WC-1 blue light photoreceptor (Froehlich et al., 2002; Levina et al., 2002) forms a complex with WC-2 (white collar complex [WCC]) that binds the *frq* promoter and directly activates transcription of the *frq* gene (Froehlich et al., 2003a). Levels of FRQ protein then slowly increase; FRQ dimerizes (Cheng et al., 2001a) and forms a complex with FRH (a FRQ-interacting RNA Helicase; Cheng et al., 2005) that binds to, and promotes the phosphorylation of, the WCC (Cheng et al., 2001a; Denault et al., 2001; He et al., 2005b; Schafmeier et al., 2005). Once hyperphosphorylated, the WCC is unable to activate transcription of *frq* (Schafmeier et al., 2005), resulting in reduced *frq* transcript levels and a decrease in FRQ protein production. FRQ also acts in a positive feedback loop, maintaining levels of *wc-2* mRNA and WC-1 and WC-2 proteins, conferring stability and robustness to the oscillator (Cheng et al., 2001b; Lee et al., 2000; Schafmeier et al., 2006). Subsequent phosphorylation-induced decay of FRQ, in conjunction with dephosphorylation of the WCC by phosphatase 2A (Schafmeier et al.,

2005), releases the inhibitory effect on the WCC and leads to reactivation of *frq* transcription, allowing the cycle to start anew (Liu et al., 2000). In addition to the core FRQ/WCC oscillator there is a growing body of evidence that suggests the circadian clock system as a whole includes other, as yet uncharacterized, oscillators (Christensen et al., 2004; Correa et al., 2003; de Paula et al., 2006; Ramsdale and Lakin-Thomas, 2000).

Time-of-day information is passed from the oscillator(s) to gene and gene products residing in output pathways to generate the overt daily rhythms. Genes that are rhythmically expressed under control of the clock, and reside downstream of the output pathways from the clock, are termed clock-controlled genes (ccgs; Loros et al., 1989). To date, over 180 *ccgs* have been identified in *N. crassa* (Bell-Pedersen et al., 1996b; Correa et al., 2003; Loros et al., 1989; Nowrousian et al., 2003; Zhu et al., 2001), for review see Vitalini et al., 2006). However, only a handful of these ccgs have been studied in depth and few details of the output pathways from the *N. crassa*, or any other organism's circadian oscillator, are known.

One of the most direct links from a circadian oscillator to a purported output pathway has been established in the cyanobacterium, *Synechococcus elongatus.* A canonical two-component system composed of the histidine kinase (HK) SasA and the response regulator (RR) RpaA receives temporal information directly from the oscillator to regulate the activation of global gene expression (Iwasaki et al., 2000; Takai et al.,

2006). More complex two-component systems, termed 'phosphorelays', exist in plants and fungi where they act as signal transduction pathways for various hormonal and environmental stress responses, and, as suggested in this study, for the circadian clock. Phosphorelay systems consist of a hybrid HK sensor protein, which is phosphorylated in response to some signal; this phosphoryl group is then transferred to a receiver domain on the hybrid kinase, and then transferred to a histidine phosphotransferase (HPT), which shuttles the phosphoryl group to a RR to elicit the appropriate response (Loomis et al., 1998). Often, the role of the RR is to modulate the activity of a downstream MAP kinase cascade, as is the case in the *Saccharomyces cerevesiae* high osmolarity glycerol (HOG) pathway and the *Schizosaccharomyces pombe* oxidative stress response pathway (Hohmann, 2002; Ikner and Shiozaki, 2005), which in turn controls downstream target genes.

Analysis of the genome sequence of *N. crassa* has revealed the existence of 11 putative HKs, one HPT, and two RRs, suggesting the presence of at least one phosphorelay system (Borkovich et al., 2004). Indeed, components of an osmosensing pathway homologous to the yeast HOG pathway have been described, including a sensor hybrid HK (*osmotic sensitive-1*, *os-1*; Schumacher et al., 1997), a MAP kinase cascade (*os-4,* MAPKKK; *os-5,* MAPKK*; os-2,* MAPK; Fujimura et al., 2003; Zhang et al., 2002) and the interceding RR (*rrg-1,* Jones et al., 2007).

The discovery of a connection between the fungal circadian clock and a phosphorelay pathway described herein initiated from a previous study in which we described a genetic selection designed to obtain mutations that lie in the output pathway(s) from the FRQ/WCC oscillator to affect expression levels of the morning-specific *ccg-1* gene (Vitalini et al., 2004). Three of the mutant strains isolated were of particular interest (COP1-2, COP1-3, and COP1-4) because, in addition to displaying constitutively low levels of *ccg-1* mRNA, they have a period defect on race tubes and an 'Eas-like' (easily-wettable spore) appearance on slants. These additional phenotypes suggested that the genes mutated in these strains function in a pathway(s) that regulates the expression of several ccgs, not just *ccg-1,* because *ccg-1* null strains display no discernable phenotypes. During the preliminary analysis of these three strains we found that the mutation in one of them, COP1-4, is linked to the *mat* locus on linkage group I (LG I), providing a valuable starting point from which to begin genetic mapping of the mutation. In this work, we show that: (1) *response regulator-1* (*rrg-1*) is the gene mutated in the COP1-4 mutant strain; (2) the *N. crassa* HOG pathway, in which *rrg-1* functions, is regulated by, and acts as an output pathway from, the circadian clock; and (3) the clock is not required to mount an acute response to hyperosmotic conditions, but circadian regulation of this pathway may allow the organism to anticipate and prepare for daily fluctuations in environmental osmolarity. Together, these results provide the first molecular description of an output pathway between the circadian clock and overt rhythmicity in *N. crassa*.

Materials and Methods

Strains and culture conditions

All strains used in this study are listed in Table 3-1. All strains contain the *bd* mutation (unless indicated otherwise), which confers resistance to elevated the CO_2 levels that occur in enclosed culture tubes (race tubes) and clarifies the circadian rhythm in conidiation without otherwise affecting clock function (Sargent and Kaltenborn, 1972). Vegetative cultures were maintained on Vogel's minimal (VM; 1X Vogel's salts, 2% glucose) medium and handled according to standard procedures (Davis, 1970). Strains carrying the *hph* cassette, which confers resistance to hygromycin, were maintained on VM supplemented with 200 μg/ml hygromycin. Strains transformed with the rescue-plasmid, pCJ2 (Jones et al., 2007), which contains the *bar* gene that confers resistance to BASTA (Bayer), were selected on VM medium lacking nitrogen and supplemented with 200 μg/ml BASTA and 0.5% proline (as a minimal source of nitrogen). Race tube, osmotic induction, and time series assays were done in environmentally-controlled chambers (Percival Scientific, Inc., Perry, IA). Race tube medium contains 1X Vogels's salts, 0.1% glucose, 0.5% arginine, and 1.5% agar. Osmotic sensitivity assays were performed on solid VM medium supplemented with 4% NaCl, 4% KCl, or 1M sorbitol. Osmotic induction experiments were performed as described previously (Shinohara et al., 2002) using liquid VM with the following modification: 5M NaCl was added directly to the culture medium to a final concentration of 0.7 M (4%). Time series experiments for analysis of protein or RNA were performed as described previously (Correa and Bell-Pedersen, 2002). *ccg-1* mRNA levels are

Table 3-1
N. crassa strains used for this study

Strain[1]	Genotype	Phenotype	Strain number	Source/Reference
Wild Type	*bd mat* a	wild-type; 22 hour period; rhythmic *ccg-1* mRNA levels	FGSC** #1859	FGSC** #1859
frq^{7}	*bd frq*^{7} *mat* a	29 hour period; rhythmic *ccg-1* mRNA levels	DBP 294	Feldman and Hoyle. 1973
Δ*frq*	*bd frq*^{10} *mat* A	arrhythmic; constitutive high *ccg-1* mRNA levels	DBP 40-2	Aronson *et al.* 1994
Δ*wc-1*	*bd* Δ*wc-1 mat* a	arrhythmic; constitutive low *ccg-1* mRNA levels	DBP 580	Lee *et al.* 2003
COP1-4	*bd cop1-4 mat* a	20 hour period; constitutive low *ccg-1* mRNA levels; osmotically sensitive	DBP 464.1	Vitalini *et al.* 2004
Δ*rrg-1*	*bd* Δ*rrg-1 mat* A	20 hour period; constitutive low *ccg-1* mRNA levels; osmotically sensitive	1053 Δ*rrg-1*	Jones *et al.* 2007
os-1	*bd os-1 mat a*	osmotically sensitive	DBP 56	
leu-3 arg-1*	*leu-3 arg-1 mat* A	leucine and arginine auxotroph	FGSC** #1210	FGSC** #1210

1. All strains carry the *bd* mutation unless otherwise indicated
*. Does not carry the *bd* mutation
**. Fungal Genetics Stock Center, University of Missouri, Kansas City, MO

much lower in the Δ*rrg-1* strain in the osmotic induction conditions than when they are examined in a time series, likely due to the increased glucose content of the medium in the osmotic induction experiments (2% vs. 0.03%) as *ccg-1* is known to be repressed in the presence of elevated glucose levels (McNally and Free, 1988).

Plasmid construction and sequencing

The entire *rrg-1* ORF, including 271 bp 5' and 512 bp 3', was amplified by polymerase chain reaction (PCR) using genomic DNA from the wild-type or COP1-4 strain as template. The resulting 4.2 kb fragments were each cloned into the pCR-Blunt II-TOPO vector (Invitrogen) to produce pMV1 and pMV2, respectively. Sequencing reactions were performed using BigDye terminator mix (Applied Biosystems) per manufacturer's instructions and analyzed at the Gene Technology Laboratory (Institute of Developmental and Molecular Biology, Texas A&M University). Sequence analysis was performed using Sequencher software version 4.2 (Gene Codes).

Nucleic acid isolation, protein isolation, and hybridization

RNA isolation and Northern hybridization protocols have been described previously (Bell-Pedersen et al., 1996a). Radioactive riboprobes were synthesized from pKL119 (*ccg-1*) or pMV1 (*rrg-1*) using T3 or T7 polymerases, respectively, in the presence of [α-^{32}P]UTP. Total protein isolation and Western hybridization were done as described in (Garceau et al., 1997) and (Jones et al., 2007) for detection of FRQ protein and OS-2

protein, respectively. Antibodies that recognize OS-2 total protein (anti-HOG-1) or only phospho-OS-2 (anti-p38) were obtained from Santa Cruz Biotechnology.

Results

response regulator-1 *is the gene mutated in the COP1-4 mutant*

Genetic mapping of the mutation in the COP1-4 strain was performed by crossing COP1-4 to a *leu-3 arg-1* double mutant strain (FGSC #1210); the *mat, leu-3,* and *arg-1* loci are all on the left arm of LG I. Analysis of the progeny from this cross placed the mutation in the COP1-4 strain in a region between the *mat* locus and *arg-1* (6.5%, 1.4%, and 2.3% recombination with *leu-3*, *mat,* and, *arg-1,* respectively; Figure 3-1A and data not shown). Examination of this region of the physical map at the *N. crassa crassa* e-compendium gene list (http://www.bioinf.leeds.ac.uk/~gen6ar/newgenelist/genes/gene_list.htm) revealed a single locus, *response regulator-1* (*rrg-1*), with mutant phenotypes similar to those of the COP1-4 strain.

The *rrg-1* ORF is predicted to encode an 1115 amino acid protein with a C-terminal receiver domain typical of those in phosphoryl-accepting RR proteins (Figure 3-1A and Jones et al., 2007). Sequencing of the *rrg-1* locus in the COP1-4 mutant strain revealed a C to T point mutation at nucleotide 2,710 of the predicted open reading frame (ORF) resulting in a premature STOP codon (codon 904). This mutation is predicted to produce an RRG-1 protein that lacks a functional receiver domain as the protein would be

truncated before encoding the aspartyl residue necessary for phosphotransfer (Figure 3-1A and Jones et al., 2007). An *rrg-1* knockout strain (Δ*rrg-1*), created by replacement of the *rrg-1* locus with the bacterial hygromycin-resistance gene (*hph*) has been reported previously (Jones et al., 2007); this strain was crossed to a *bd* strain to obtain the *bd* Δ*rrg-1* strain (referred to as Δ*rrg-1* from here on). To further characterize the nature of the COP1-4 mutation and confirm its identity as the causative agent of the observed phenotypes, we analyzed the phenotypes of both the COP1-4 mutant strain and the Δ*rrg-1* strain under a variety of conditions.

Both the COP1-4 strain and the Δ*rrg-1* strain display an "Eas-like" appearance on minimal slants (Figure 3-1B), and a one-hour period shortening on race tubes as compared to the wild-type strain (Figure 3-1C). Additionally, both the COP1-4 and Δ*rrg-1* strains show a delay in conidial band formation upon transfer to DD conditions. The wild-type strain forms a full conidial band the first day in DD and each day thereafter; the COP1-4 strain conidiates very little or not at all the first day in DD but conidiates normally each day thereafter, and the Δ*rrg-1* strain does not form normal conidial bands until the third day in DD (Figure 3-1C). Because of the reported sensitivity of the Δ*rrg-1* strain to hyperosmotic conditions as a consequence of its role in the HOG pathway (Jones et al., 2007), we also examined the COP1-4 mutant for osmotic sensitivity by inoculation onto VM media supplemented with different osmolytes. The wild-type strain is able to grow on all media examined, whereas a known osmotically sensitive mutant (*os-1*) is able to grow only on media without an osmolyte

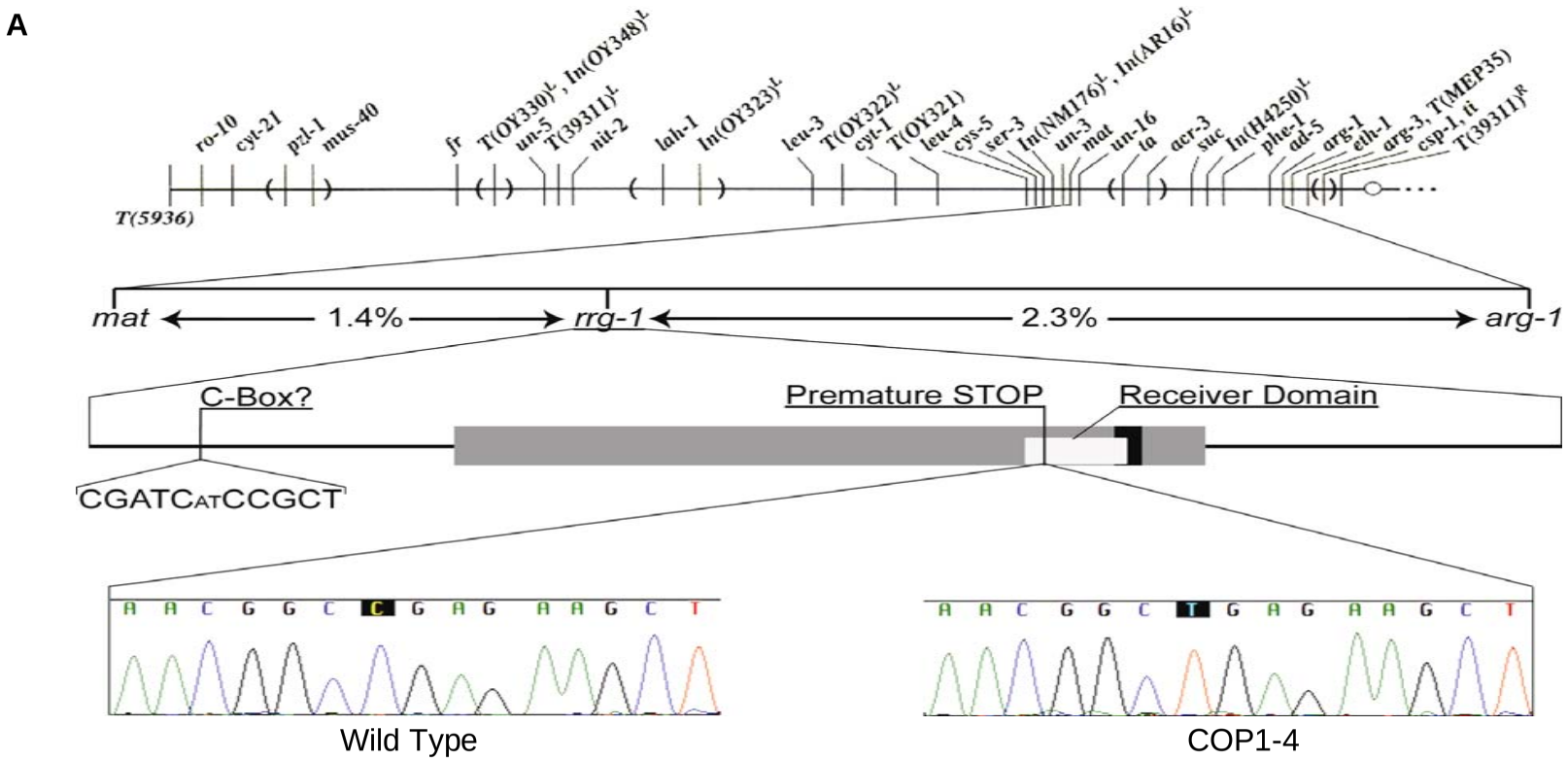
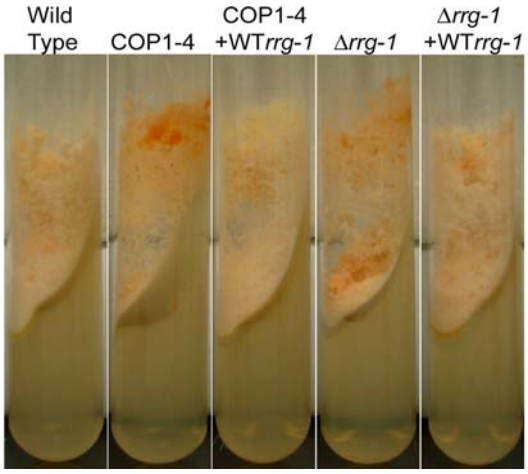


Figure 3-1. The COP1-4 strain contains a mutation in the *rrg-1* locus. (A) Genetic map of the left arm of LG I (from the 2001 *Neurospora* Compendium) showing the relative positions of selected loci (top) and a blow-up of the region between the *mat* and *arg-1* loci where the mutation in the COP1-4 strain is located (top). In the schematic of the *rrg-1* locus (middle), the ORF is represented by the medium grey box, a single 85 bp intron is indicated by the small, black box, and the region predicted to encode the receiver domain is indicated by the light grey box. A near-consensus WC-1 binding site and putative clock-box is located 1.5 kb upstream from the predicted translational start site. The relative position of the point mutation in the COP1-4 strain is indicated. In the sequence chromatograph of the region of the *rrg-1* locus that is mutated in the COP1-4 strain (bottom), a C-T transition results in a premature STOP (TGA) codon where a codon for arginine (CGA) occurs in the wild-type strain.

B



C

Figure 3-1 Continued.

(B) Phenotype of the COP1-4 strain, the Δ*rrg-1* strain, and these strains transformed with plasmid pCJ2 (COP1-4 +WT*rrg-1* and Δ*rrg-1* +WT*rrg-1*) on slants. (C) Both the COP1-4 strain and the Δ*rrg-1* strain display a 1 hour period defect on race tubes. The yellow and black bar indicates time in LL and DD, respectively. Each strain was inoculated and grown in LL for 24 hours before transfer to DD, after which time the growth front was marked every 24 hours (black lines). To more clearly visualize the period differences, the second- and third-to-last growth front markings have been erased. The period of each strain in hours +/- standard error of the mean is presented.

D

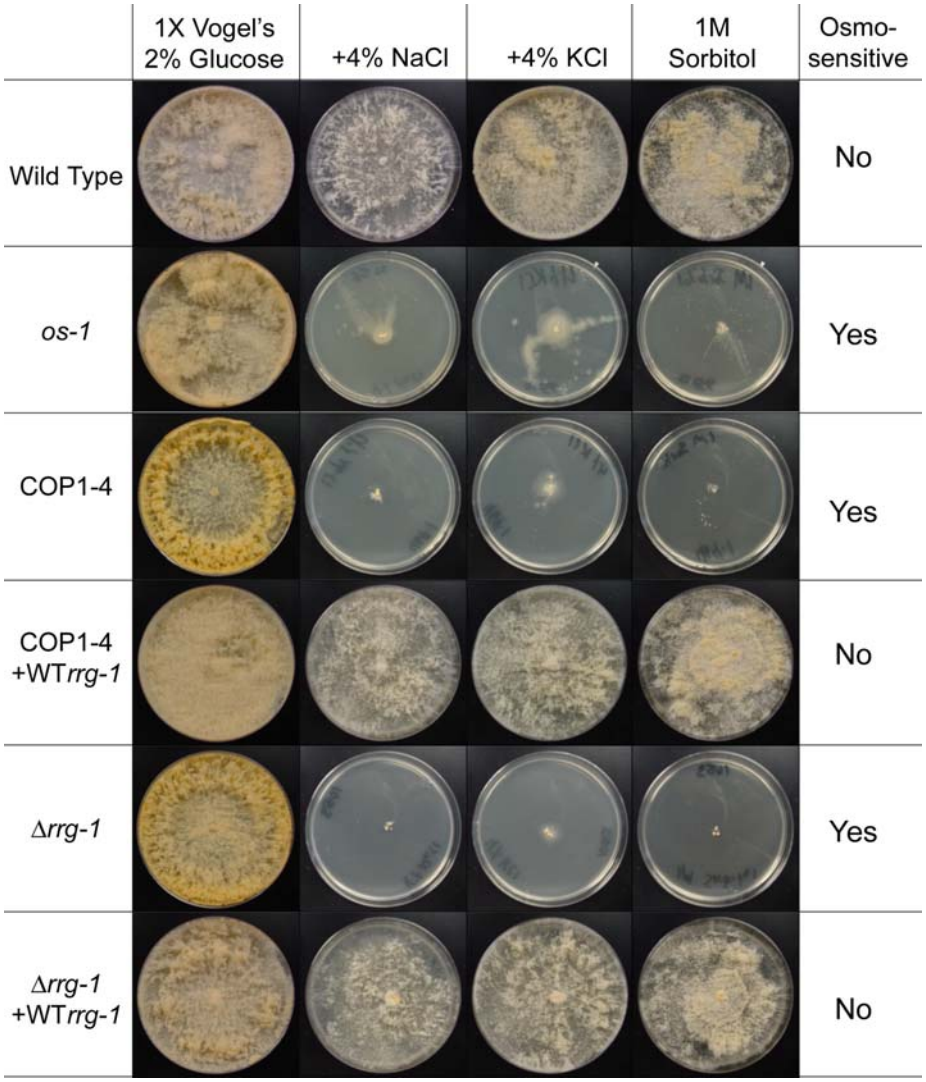


Figure 3-1 Continued.

(D) The COP1-4 and Δ*rrg-1* strains are osmotically sensitive. Strains were inoculated onto minimal medium, or the same medium supplemented with the indicated osmolyte. Strains are identified on the left, media are given at the top, and osmotic phenotype is indicated at right.

supplement (Figure 3-1D). Both the COP1-4 mutant strain and the Δ*rrg-1* strain phenocopied *os-1*; only media without an osmolyte supplement supported their growth (Figure 3-1D). Each of these phenotypes is identical in both the COP1-4 mutant strain and the Δ*rrg-1* strain and each phenotype is rescued in either strain following transformation with plasmid pCJ2, which contains the wild-type *rrg-1* locus (Figure 3-1B, C, D, and Jones et al., 2007). These data indicate that *rrg1* is the locus mutated in the COP1-4 strain and that the COP1-4 mutation phenocopies the null allele of *rrg-1* under the conditions tested.

The FRQ/WCC oscillator is functional in the Δrrg-1 *strain*

In a wild-type strain, levels of *ccg-1* mRNA accumulate in a circadian fashion, with peak levels occurring around 12 hours after entering constant dark (DD12) and a trough in *ccg-1* mRNA levels occurring around DD24 (Loros et al., 1989). We previously showed that in DD, *ccg-1* mRNA levels are arrhythmic and constitutively high in a Δ*frq* strain (Figure 3-2A and Vitalini et al., 2004), and arrhythmic and constitutively low and in the COP1-4 mutant strain (Vitalini et al., 2004). Analysis of the Δ*rrg-1* strain confirmed this phenotype: *ccg-1* mRNA levels are arrhythmic and constitutively low at all times of day (Figure 3-2A and data not shown). A similar phenotype was observed in a Δ*wc-1* strain: levels of *ccg-1* mRNA remain low over the course of a day (Figure 3-2A). If *rrg-1* is solely part of an output pathway from the clock, as these data suggest, we would predict that the absence of *rrg-1* would not affect the function of the

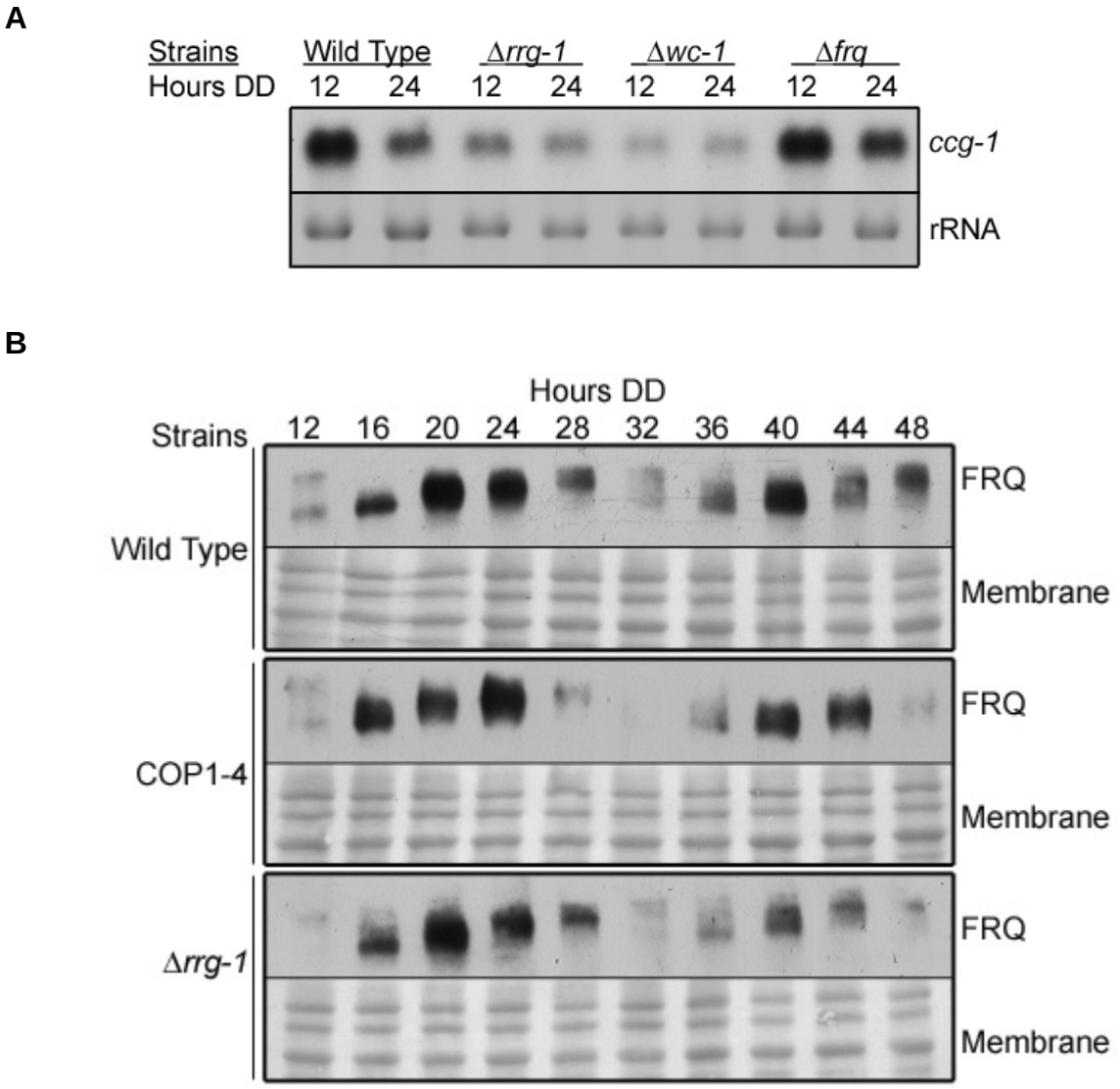


Figure 3-2. The FRQ/WCC oscillator is functional, but unable to drive rhythmic accumulation of *ccg-1* mRNA, in *rrg-1* mutant strains. (A) Northern blot showing levels of *ccg-1* mRNA at DD12 and DD24 in selected strains. Hours in DD and strains are indicated above the blot. The ethidium bromide stained gel is shown as a loading control. (B) Western blot of FRQ protein in selected strains. Hours in DD are shown above the blots and the strains are indicated at left. Amido Black stained membranes are shown as loading controls.

FRQ/WCC oscillator, only the ability of this oscillator to transduce temporal information through the *rrg-1*-based output pathway to drive rhythmic accumulation of *ccg-1* mRNA. To test this hypothesis we examined the levels of FRQ protein accumulation over a 48 hour period in DD. As predicted, the accumulation of FRQ protein is rhythmic in both the COP1-4 mutant strain and the Δ*rrg-1* strain (Figure 3-2B). Together these data indicate that functional *rrg-1* is not necessary for the core FRQ/WCC oscillator to operate properly, but is required for rhythmic accumulation of *ccg-1* mRNA.

The N. crassa *HOG pathway is regulated by the circadian clock*

Because *rrg-1* is required for circadian control of *ccg-1* mRNA levels, but not FRQ/WCC oscillator function, we predicted that: (1) *rrg-1* lies in an output pathway from the FRQ/WCC oscillator, and (2) the HOG pathway, in which *rrg-1* is known to function (Jones et al., 2007), may also act as a circadian output pathway. We reasoned that if the HOG pathway is an output pathway from the FRQ/WCC oscillator, at least one component of this pathway would be regulated in a circadian manner under constant conditions. We used the consensus WC-1 binding site sequence (CGATCnCCGCT; (Froehlich et al., 2002) to search for similar sequences in the region upstream of the *rrg-1* ORF. We discovered one of these sites 1.5 kb upstream from the predicted translational start site. This placement is similar to that of the clock box in the *frq* promoter, which is bound by the WCC and is necessary and sufficient to produce rhythms in *frq* mRNA accumulation (Figure 3-1A; Froehlich et al., 2003a). This finding suggested the possibility that the expression of *rrg-1* may be controlled directly by

oscillator components, specifically the WCC. We examined *rrg-1* mRNA levels in the long period frq^7 strain to determine whether *rrg-1* transcript accumulates rhythmically in DD conditions. Strains carrying the frq^7 mutation display more robust rhythms in transcript accumulation than wild-type strains (Bell-Pedersen et al., 1996a) and thus, even a low-amplitude rhythm in mRNA abundance is more easily detected in this strain. We found that steady-state *rrg-1* mRNA levels fluctuate but do not display a detectable circadian rhythm as compared to the control *ccg-1* transcript, which displays a robust rhythm in mRNA accumulation (Figure 3-3). Similar results were obtained using the wild-type strain (data not shown).

Given the lack of rhythmicity in *rrg-1* mRNA abundance, and that signal transduction through the HOG pathway involves the transfer of a phosphoryl group from one protein component of the pathway to the next (for review see Hohmann, 2002), we examined the possibility that the clock signal through the HOG pathway is at the level of protein accumulation and/or modification. Recall that in the HOG pathway RRG-1 regulates the activity of a MAP kinase cascade in which OS-2 protein is the MAP kinase, and phosphorylation of OS-2 is required for its function. Due to the availability of commercially-produced antibodies that differentially recognize only phosphorylated OS-2 protein (phospho-OS-2) or total OS-2 protein (both phosphorylated and unphosphorylated; Jones et al., 2007), we examined the accumulation and phosphorylation state of this protein over the course of two days in DD conditions. We

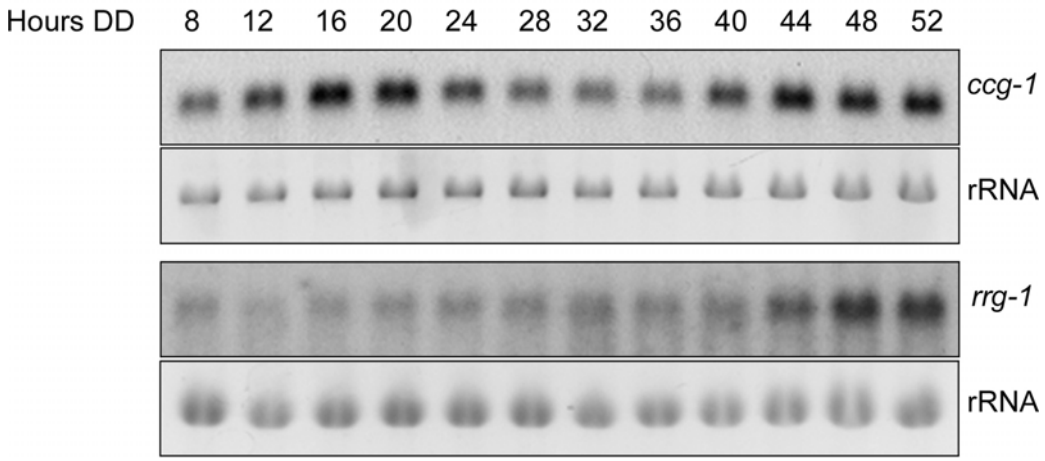


Figure 3-3. The steady-state levels of *rrg-1* mRNA are arrhythmic in the high-amplitude *frq*^{7} strain. Northern blots of *ccg-1* and *rrg-1* mRNA levels are shown. Time in DD is given across the top. The ethidium bromide stained gels are shown as loading controls.

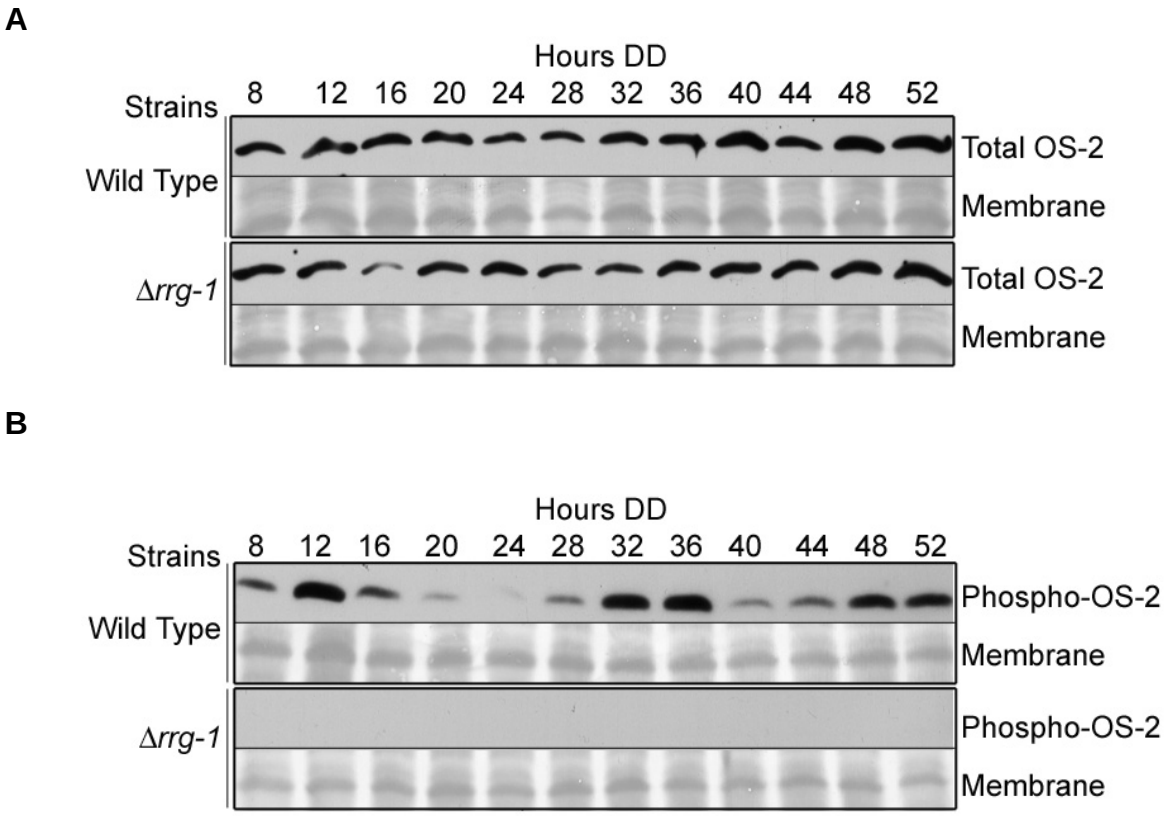


Figure 3-4. Phosphorylation, but not accumulation, of OS-2 protein displays a circadian rhythm in *N. crassa*. (A) Western blots showing accumulation of total (both phosphorylated and unphosphorylated) OS-2 protein and (B) Western blots showing accumulation of phosphorylated OS-2 protein. In both panels Time in DD is indicated at the top and strains are indicated at left. Amido Black stained membranes are shown as loading controls.

found that the steady-state levels of total OS-2 protein remain fairly constant over the course of the day in both the wild-type and Δ*rrg-1* strains (Figure 3-4A). Conversely, there is a robust circadian rhythm in the levels of phospho-OS-2 in the wild-type strain (Figure 3-4B). This phosphorylation is completely absent in the Δ*rrg-1* (Figure 3-4B) and COP1-4 mutant strains (data not shown).

In order to demonstrate that the rhythmic phosphorylation of OS-2 is a result of input to the HOG pathway from the FRQ/WCC oscillator, we examined levels of total and phospho-OS-2 in DD in strains lacking FRQ or WC-1. We found that levels of total OS-2 protein are comparable in the wild-type and Δ*frq* strains; however, there is a severe reduction in the levels of total OS-2 protein in the Δ*wc-1* strain (Figure 3-5A). Additionally, phospho-OS-2 is absent at all times of day in the Δ*wc-1* strain, and present at all times of day in a Δ*frq* strain (Figure 3-5B). The levels of phospho-OS-2 and *ccg-1* mRNA observed in these strains supports the idea that the HOG pathway connects the FRQ/WCC oscillator to the expression of *ccg-1*. In the wild-type strain, levels of phospho-OS-2 are rhythmic with peak levels occurring at about the same time of day as peak *ccg-1* mRNA levels (Figures 3-2A and 3-4B; Loros et al., 1989). Strains with constitutively low (or no) phosphorylated OS-2 (Δ*rrg-1* and Δ*wc-1*) also display constitutively low levels of *ccg-1* mRNA (Figures 3-2A and 3-5); conversely, the Δ*frq* strain, which displays constitutively elevated levels of phospho-OS-2, also displays constitutively elevated levels of *ccg-1* mRNA (Figures 3-2A and 3-5).

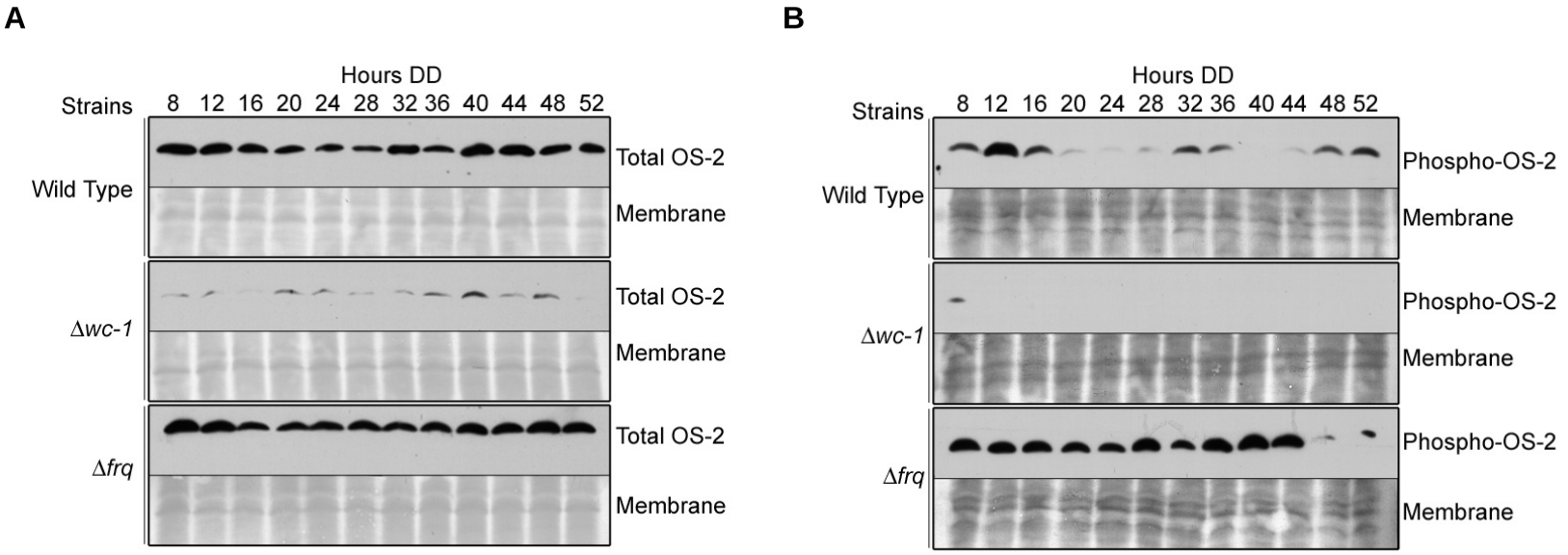


Figure 3-5. Rhythmic accumulation of phosphorylated OS-2 protein is dependent upon the FRQ/WCC oscillator. Western blots showing amounts of total (A) or phosphorylated (B) OS-2 protein in selected strains. Time in DD is shown at top, strains are indicated at left. Amido Black stained membranes are shown for loading controls.

The FRQ/WCC oscillator is not necessary for the osmotic-stress response

Both the phosphorylation of OS-2 protein and the accumulation of *ccg-1* mRNA are induced in response to hyperosmotic stress (Jones et al., 2007; Lindgren, 1994) and are also regulated by the circadian clock (Figure 3-4B and 3-5; Loros et al., 1989). The circadian regulation of these events requires an intact FRQ/WCC oscillator and HOG pathway (*i.e.,* a functional copy of *rrg-1*), and osmotic induction of OS-2 phosphorylation requires *rrg-1* (Jones et al., 2007). We next investigated whether the osmotic-stress response is dependent upon components of the circadian clock; that is, does the regulation by the clock overlap with the osmotic-stress related regulation of this pathway? As has been reported previously, phospho-OS-2 and *ccg-1* mRNA, but not total OS-2 protein levels, are induced in response to treatment with 4% NaCl in a wild-type strain (Figure 3-6A, B and C; Jones et al., 2007;Lindgren, 1994); Phospho-OS-2 is detectable within five minutes, and *ccg-1* mRNA accumulates significantly within one hour after the introduction of 4% NaCl. The sustained induction of phospho-OS-2, and any induction of *ccg-1* mRNA, by 4% NaCl is dependent upon *rrg-1* (Figure 3-6B and C; Jones et al., 2007). Phospho-OS-2 and *ccg-1* mRNA levels are both induced in response to the presence of 4% NaCl in the clock-mutant Δ*frq* and Δ*wc-1* strains in a manner similar to the wild-type strain (Figure 3-6B and C). These results are consistent with the osmotic sensitivity phenotypes of these strains observed on solid medium supplemented with various osmolytes; the Δ*rrg-1* strain is unable to grow in conditions of osmotic stress whereas the wild-type and clock-mutant strains display no such sensitivity (Figure 3-1D and data not shown). These data suggest that regulation of the

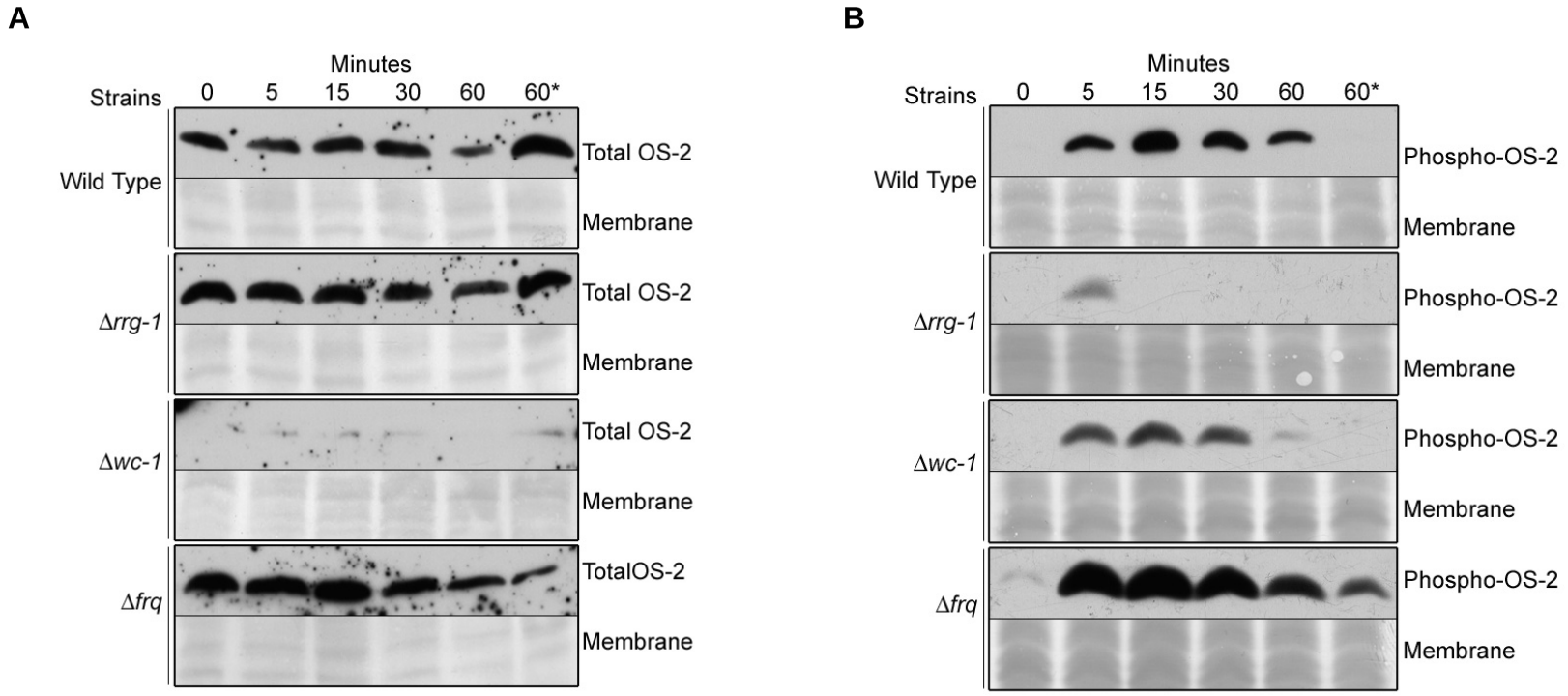


Figure 3-6. *rrg-1* is, but *frq* and *wc-1* are not, necessary for osmotic induction of phospho-OS-2 protein and *ccg-1* mRNA levels. Western blots showing total (A) or only phosphorylated OS-2 protein (B) in response to hyperosmotic conditions. Strains are given at left and time in the presence of 4% NaCl is given at the top; the asterisks indicate treatment with sterile distilled water instead of 4% NaCl as a control. Amido Black stained membranes are shown for loading control.

C

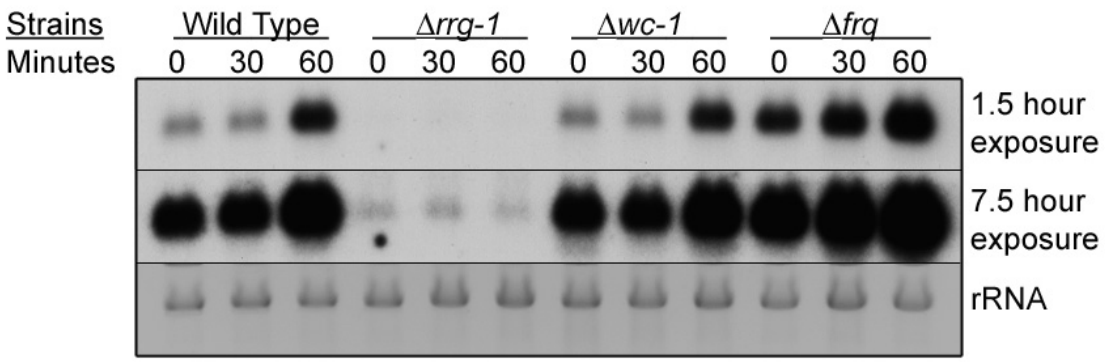


Figure 3-6 Continued.

(C) Northern blot of *ccg-1* mRNA levels in response to hyperosmotic conditions. Strains and Time in 4% NaCl are given across the top, and length of film exposure is shown to the right. The ethidium bromide stained gel is shown as a loading control.

HOG pathway by osmotic stress and regulation by the circadian clock are separate processes and occur through different upstream pathways.

Discussion

We previously described a selection for mutations in *N. crassa* that affect the expression of a clock-controlled gene, *ccg-1,* and lie in an output pathway from the circadian clock. In some of the mutant strains obtained, the observed phenotypes suggested that the expression of more than one ccg was affected, and thus, that the mutations are in genes that lie just downstream of the core circadian oscillator. In the present study we describe the identification of one of the mutated genes, *rrg-1*, as the causative agent of the observed circadian phenotypes in strain COP1-4, and as a component of a signaling pathway (the HOG pathway) that links the circadian clock to downstream ccgs. This study represents the first description of a circadian output pathway at the molecular level and has provided insight into one mechanism by which the clock regulates the expression of downstream genes to affect cellular processes.

A common pathway serves as both a regulator of osmotic-stress-induced responses and a circadian-output pathway

The original COP1-4 mutant strain and the Δ*rrg-1* strain display nearly identical phenotypes under all conditions examined, confirming that COP1-4 harbors a loss-of-function mutation that results in a null-allele phenotype (Figure 3-1B, C, and D). We had previously predicted that the gene mutated in COP1-4 functions in an output pathway

from the clock and is not part of the central oscillator based on the observation that, although *ccg-1* mRNA levels are constitutively low, this strain still displays rhythmic conidiation on race tubes, albeit with a slight period defect and delayed band formation. Mutations in components of the central FRQ/WCC oscillator display more severe race tube phenotypes ranging from over two-hour period defects to complete arrhythmicity (for review see Liu and Bell-Pedersen, 2006). The identification of *rrg-1* as the mutated gene in COP1-4 further supported this idea, as *rrg-1* is known to function in a signal transduction pathway, which, until this study, was not known to interact with the circadian clock. Examination of FRQ protein levels in DD confirmed that the FRQ/WCC oscillator is functional in the absence of *rrg-1*, although it is unable to drive rhythmic *ccg-1* mRNA accumulation. However, the short period phenotype on race tubes suggests the possibility of feedback from this output pathway, or from a downstream target(s) of this pathway, to the oscillator itself. It should be noted that although normal conidial bands are not produced for the first one to two days in DD in the *rrg-1* mutant strains, bands of aerial hyphae do occur in a rhythmic fashion. Additionally, this delay in band formation is not seen when the strains are permitted to grow further down the race tube before entering DD (data not shown). Together, these observations suggest that the delay is not truly a circadian phenotype but is related to some other aspect of HOG pathway function.

rrg-1 was previously demonstrated to act in the HOG pathway, which is responsive to osmotic stress, and the circadian phenotypes observed suggested that this same pathway

could also function in output from the circadian clock. The HOG pathway in *S. cereviseae* has been well characterized and is known to control a wide range of downstream genes through activation of both positive and negative transcription factors by the HOG-1 MAP kinase (for review see Hohmann, 2002). Consistent with the *S. cerevisea* data, many homologous genes are regulated by the OS-2 MAP kinase in *N. crassa* (Noguchi et al., 2007) and mutations in components of the HOG pathway (*rrg-1*) display multiple phenotypes in addition to osmotic sensitivity and the circadian regulation defects described here (*e.g.*, female sterility and loss of conidial integrity, Jones et al., 2007). By regulating the activity of this homologous pathway during the daily cycle, the *N. crassa* clock could influence the expression of a multitude of downstream genes through control of OS-2-regulated transcription factors. Our data indicate that the phosphorylation, and thus activity, of the OS-2 MAP kinase of this pathway is indeed regulated in a circadian manner (Figure 3-4B). Interestingly, the level of *ccg-1* transcript appears to directly correlate with the presence of phospho-OS-2 protein. The Δ*rrg-1* and Δ*wc-1* strains, which lack phospho-OS-2 protein, have relatively lower levels of *ccg-1* transcript as compared to wild type; the Δ*frq* strain displays both constitutive levels of phospho-OS-2 protein and elevated levels of *ccg-1* mRNA compared to the wild-type strain (Figures 3-2A and 3-5). Additionally, the time of day when levels of phosphorylated OS-2 protein are highest in the wild-type strain is about the same time at which *ccg-1* transcript is most abundant (Figures 3-2A and 3-4B; Loros et al., 1989). The delay between the induction of phospho-OS-2 (within five minutes) and the induction of *ccg-1* mRNA (within 30-60 minutes) in response to an

osmotic stress (4% NaCl) is suggestive of indirect regulation of *ccg-1* by the OS-2 MAP kinase (Figure 3-6). These results suggest that the clock regulates the activity of the HOG pathway, thereby imposing circadian regulation of downstream targets of this pathway (transcription factors), with the end result being circadian regulated gene expression (*i.e.*, *ccg-1* mRNA).

Regulation of the HOG pathway by osmotic stress does not require the clock components FRQ or WC-1

The discovery of *rrg-1*-dependent clock regulation of the HOG pathway prompted us to investigate whether osmotic-stress regulation of this pathway required components of the FRQ/WCC oscillator. We did find that the levels of total OS-2 protein are severely compromised in the Δ*wc-1* strain (Figures 3-5A and 3-6A); however, these levels are sufficient for this strain to mount an acute response to osmotic stress (Figure 3-6B and C). Regardless of the levels of phospho-OS-2 observed in the clock mutant strains under non-inducing conditions, there is a robust induction of both phospho-OS-2 and *ccg-1* mRNA in both the Δ*frq* and Δ*wc-1* strains upon exposure to a hyperosmotic medium (Figure 3-6B and C). These data suggest that input of information regarding the osmolarity of the environment, and input of time-of-day information into this pathway, occur through different upstream regulators of the HOG pathway.

As reported previously (Jones et al., 2007), there is a transient and comparatively weak induction of phospho-OS-2 in the Δ*rrg-1* strain in the presence of 4% NaCl; however, it

is not sufficient to allow growth on hyperosmotic medium or activate *ccg-1* mRNA levels (Figure 3-1D and 3-6B). This phosphorylation suggests that either an alternate pathway, or an alternate RR (*N. crassa* is predicted to encode two RRs, RRG-1 and RRG-2, Borkovich et al., 2004) may be capable of activating this pathway to some extent.

Where does the clock impinge on the HOG pathway

Both circadian-clock and osmotic-stress input to this pathway occur somewhere upstream of the MAP kinase cascade (involving OS-4, OS-5 and OS-2), as both forms of regulation are absent in the COP1-4 mutant and Δ*rrg-1* strains (Figure 3-4B and data not shown; Jones et al., 2007). Precisely where the clock inputs into this pathway is not yet known. The *os-2* promoter contains two putative clock-boxes, and the *os-2* transcript was found to be rhythmic by microarray (Correa et al., 2003). WC-1, perhaps through its function in the WCC, is required for full expression of OS-2 protein; however, it is the phosphorylation of OS-2 protein that displays a circadian rhythm, which implies that additional levels of regulation by the clock must be present. In the osmotic stress response, an HPT protein (encoded by *hpt-1*) and a sensor HK (encoded by *os-1*) lie upstream of RRG-1 in this pathway (Schumacher et al., 1997; Jones et al., 2007). It is possible that clock input into the HOG pathway is through RRG-1, one of the upstream targets (OS-1 or HPT-1), or an as-yet-unidentified protein. At present, there are no antibodies available to examine the accumulation or phosphorylation of RRG-1 or other components upstream of the OS-2 MAP kinase cascade; however, the development of

these antibodies will greatly aid in the elucidation of the point at which the clock impinges upon the HOG pathway. In addition to *os-1*, the *N. crassa* genome is predicted to encode 10 other HK proteins (Borkovich et al., 2004), the functions of which are only now beginning to be investigated. It is tempting to predict that one of these other HKs could be regulated by the clock and act as a circadian output kinase to transduce time-of-day information to the HOG pathway.

It should be noted that circadian signaling through this pathway may not translate into universal regulation of all targets of the HOG pathway by the clock. As mentioned, osmotic-stress-related regulation of the HOG pathway is independent of circadian-clock regulation and these two different inputs into this pathway may function to regulate the expression of different subsets of downstream genes. In fact, over-activation of the HOG pathway can be toxic to cells (Zhang et al., 2002); consequently, the circadian output function of this pathway may differ significantly from the osmotic stress function. Alternatively, regulation of the HOG pathway may serve to prepare the organism for the daily hyperosmotic conditions that would be associated with desiccation by the sun in a natural environment. Up-regulation of the activity of this pathway, or a subset of the components necessary for that activity, may allow a preparatory response, or the potential to mount a faster response, at one time of day (morning) versus another (night). Independent regulation of this pathway by not only osmotic stress but also the clock would allow the organism to respond to unexpected stresses that occur at an unusual time of day. If hyperosmotic conditions are encountered when the clock has down-

regulated the pathway, the stress would override the clock input and allow a response to occur out of phase with the time at which it would be activated by the clock. Indeed, the osmotic induction of *ccg-1* mRNA conducted in this, and previous studies (Lindgren, 1994; Shinohara et al., 2002), is examined at DD24, corresponding to the time of day when phospho-OS-2 and *ccg-1* mRNA levels are at their lowest point in the circadian cycle. It will be interesting to examine whether the response at other times of day is comparable to the response seen here.

MAP kinase pathways as candidates for additional circadian output pathways

MAP kinase cascades, such as in the HOG pathway, are signal transduction pathways vital to environmental sensing and cellular function in fungi, and in eukaryotic systems in general. In fungal systems, MAP kinase pathways function in relaying environmental information regarding everything from the presence of a potential mate (pheromone reception), to the presence (or absence) of various stresses (*i.e.*, oxidative or osmotic stress; for review see Bahn et al., 2007). In response to these signals, MAP kinases influence the activity of numerous transcription factors, other kinases, and enzymes, which, in turn, regulate developmental, mating, and stress-response programs (Bardwell, 2006). Such far-reaching regulatory pathways are ideal candidates for output pathways from the circadian clock because, in the absence of environmental stresses, the circadian clock acts as the master regulatory system of the cell, controlling a broad range of cellular processes. Impinging on the MAP kinase regulatory pathways that control these processes would allow the clock to coordinate and regulate the majority of the

cellular environmental responses, and thus, ready these responses for the time of the day at which they are most likely to be necessary. This scenario may extend to circadian output in mammals, where MAP kinase cascades have been implemented in the regulation of cell proliferation, differentiation, development, and immune and stress responses (for review see Qi and Elion, 2005) which also have circadian components to their regulation.

Figure 3-7 depicts our current model of how this pathway fits into the circadian regulatory system. In the absence of osmotic stress, the clock activates the HOG pathway, through some as-yet-unidentified protein, during the late night to early morning. This activation of the HOG pathway leads to rhythmic phospho-OS-2 protein levels and results in circadian-regulated gene expression. Hyperosmotic stress at any time of the day can override the circadian-clock regulation of this pathway, resulting in an acute response to environmental conditions. The data presented here suggest a scenario for the first molecular description of an output pathway from the *N. crassa* circadian clock. Further investigation into the role of MAP kinase pathways in circadian output, and the transmission of time-of-day information from the clock to these pathways, will signify a major step forward in the description of circadian systems as a whole.

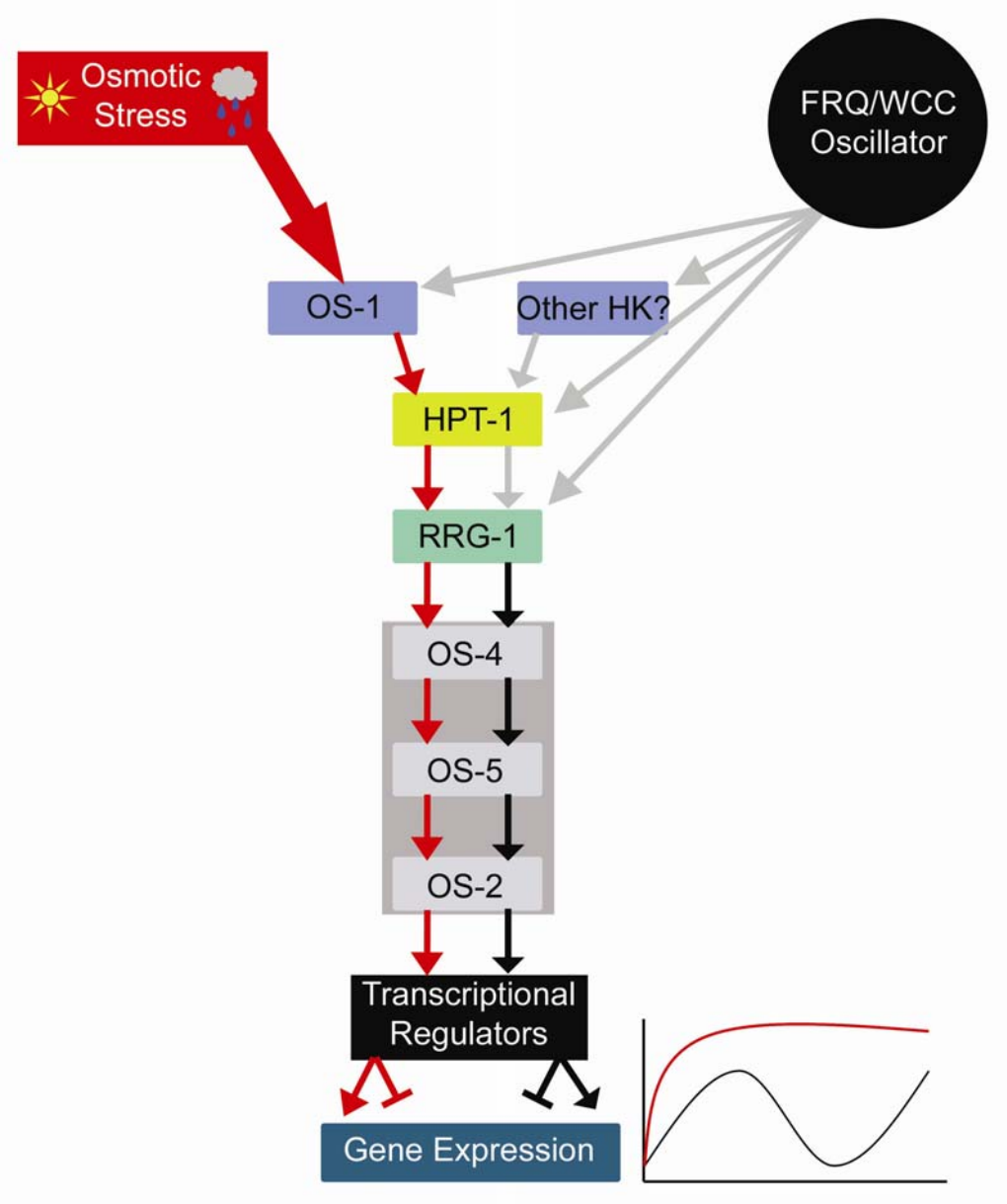


Figure 3-7. Model of the flow of osmotic-stress information, and time-of-day information, through the HOG pathway. Arrows represent flow of information only: grey arrows indicate potential points of input to the HOG pathway from the clock, red arrows indicate transduction of osmotic stress information, and black arrows indicate transduction of time-of-day information from the clock. The cartoon trace at the bottom right represents gene expression regulated by the circadian clock (black) versus osmotic stress (red) as a result of the passage of the respective information through the HOG pathway.

CHAPTER IV

CONCLUSIONS

Elucidating the mechanism of circadian clocks is an important goal because of the ubiquity of clocks and their role in many organisms' lives, including humans. The past few years have seen enormous advances in our understanding of the mechanisms of circadian oscillators, and we are now poised to begin filling in the pieces of the output pathways. While different organisms use their clocks to regulate different biological processes at different phases during the day, several fundamental aspects of rhythmicity are conserved. Thus, we expect that elucidating the output system in *N. crassa* will help us understand the workings of the circadian clock system in more complex organisms. To that end, the work presented in this dissertation represents a milestone in the continuing effort to describe and understand the output pathways of circadian systems in *N. crassa*, and organisms in general.

First Things First

The genetic selection described in Chapter II marks the first selection for mutations that affect regulation of clock-controlled genes, and the first gene to be identified from this selection (*rrg-1*, Chapter III) has resulted in the first description of a circadian output pathway (the HOG pathway) in *N. crassa*. The finding that the HOG pathway, known to function in the osmotic stress response, is also regulated by the clock, has provided insight into how output from the clock is integrated into multiple and diverse cellular processes. The data presented in this dissertation support the hypothesis that the clock is

able to impact the expression of a multitude of genes, and thereby cellular processes, by impinging on already-established regulatory pathways. The implications of these data are that 1) additional output pathways from the *N. crassa* clock may be identified through the continued characterization of mutations obtained by the described selection; 2) the clock regulates the osmotic-stress response pathway in *N. crassa*, which may provide an adaptive advantage by preparing the organism for the daily desiccation associated with exposure to the Sun; and more generally, 3) the pathways involved in transducing time-of-day information from the clock to the rest of an organism need not be clock-specific.

A Genetic Selection for Circadian Output Pathway Mutations: The Search Continues

Prior to the study presented in Chapter II, mutations affecting circadian regulation of genes and pathways in *N. crassa* (and other systems) had been obtained primarily through "brute force" screens involving thousands of strains. The description of a genetic selection for mutations that specifically affect the expression of a clock-controlled gene provides a means to more efficiently obtain mutations that lie in circadian output pathways. The mutant strains described in Chapter II represent a very small sampling of the strains acquired through this selection. The first strains pursued were chosen based on their very obvious and tractable phenotypes; however, further characterization of other mutant strains obtained will likely uncover additional components involved in circadian output. Because it is possible to select both for and

against MTR function based on media conditions, this same selection scheme can be immediately employed to search for suppressor mutations. Additionally, as more ccgs are better characterized, this selection may be adapted to look for components involved in their circadian regulation; other regulatory pathways may be uncovered through the substitution of the *ccg-1* or *ccg-2* promoter with the promoter of a different clock-controlled gene(s) (*e.g.*, *ccg-16*) as the element driving the selectable marker (*mtr*). Together, these approaches to the continued use of this selection hold promise for the further identification of additional circadian output pathways in *N. crassa*.

There are limitations to this selection that should be noted. First, although the selection is designed to uncover mutations that affect the expression of ccgs, it has been successful only under conditions where clock control of these ccgs is absent (*i.e.*, in a Δ*frq* background). This is because the selection requires the constitutive expression (either high or low) of the selectable marker. Efforts to use this selection scheme in a wild-type *frq* background have been problematic and may require further adjustment of the media and environmental conditions (*i.e.*, LL vs. DD) in order to be successful. Additionally, selection for mutations affecting *ccg-2* expression was considerably more problematic, and less successful, than the *ccg-1* targeted selection. This may be due in part to the fact that the *ccg-2* selection requires a mutation resulting in increased expression from the *ccg-2* promoter, in effect a gain-of-function mutation, to be successful. The large discrepancy in the number of mutant strains obtained by the *ccg-2*-based selection, as compared to the *ccg-1*-based selection, may be a reflection of the inherent difficulty in producing mutations that result in a gain of function, as opposed to loss-of-function

mutations that result in decreased gene expression. Even with these shortcomings, this selection will remain a useful tool in the search for additional circadian output pathways.

A Common Pathway for Osmotic-Stress and Circadian Clock Regulation of *ccg-1*

The identification of *rrg-1* as the mutated gene in the COP1-4 strain, and the dual regulation of the HOG pathway (in which *rrg-1* functions) by osmotic stress and the circadian clock, are described in Chapter III. It was known prior to this study that osmotic stress induces *ccg-1* expression; however, the connection between the HOG pathway and the circadian clock had not been previously established. The data in Chapter III show that the phosphorylation, and thus the activity, of the MAP kinase of the HOG pathway is regulated in a circadian manner, and is necessary for both the circadian and osmotic-stress-related regulation of *ccg-1* mRNA levels. There are a couple of possible interpretations of these data. One possibility is that the HOG pathway (or a subset of its components) has two independent functions: transmission of osmotic-stress information and transmission of time-of-day information (Figure 4-1A). Alternatively, the clock may regulate the entire osmotic-stress-response pathway in a time-of-day specific manner (Figure 4-1B).

MAP kinase pathways are ubiquitous in eukaryotic systems and they have been studied extensively in a number of model systems. In yeast, three parallel MAP kinase cascades have been well characterized, which together are responsible for regulating the integrity

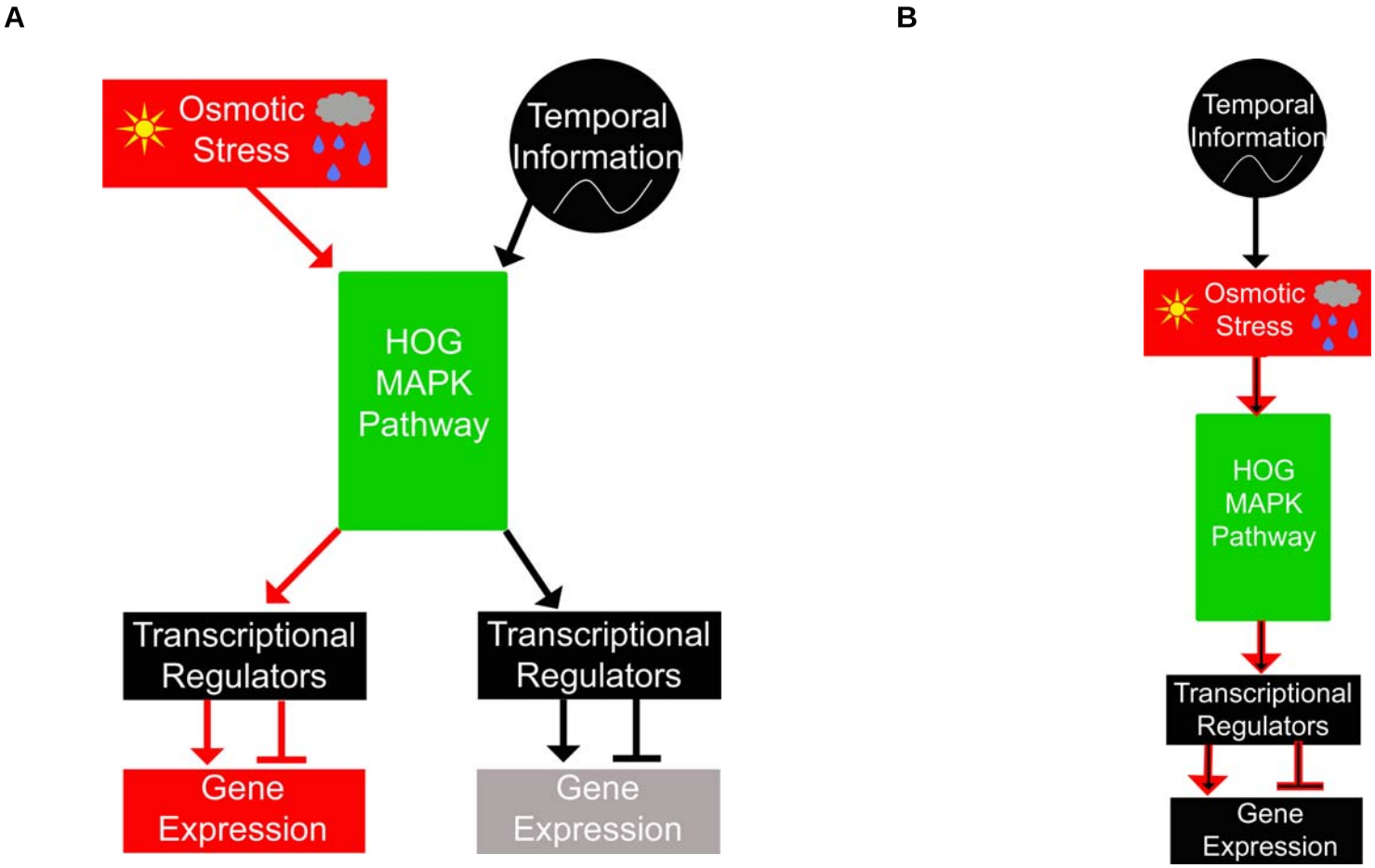


Figure 4-1. Independent roles of the HOG pathway as an osmotic-stress response pathway and a circadian output pathway versus circadian regulation of the osmotic-stress responsive genes. Red arrows indicate the transmission of osmotic-stress information, black arrows indicate time-of-day information. See text for details.

of the cell wall, mating behavior, filamentous growth, and osmotic and other stress responses (Bardwell, 2006). Interestingly, while these pathways respond to different signals, and result in the activation of different downstream genes, all three share common components; the specificity of the response is partly a function of the upstream signal that activates a given pathway (Bardwell, 2006). For example, the RR Ste20 and the MAP kinase kinase kinase (MAPKKK) Ste11 both function in all three pathways; however, different downstream effectors are activated depending on whether the upstream signal is a pheromone, a filamentous growth signal, or osmotic or other stress (Bardwell, 2006). These data support the hypothesis that the components of the HOG pathway may serve different functions, and regulate different activities, based on whether the signal into the pathway is from the clock or from an environmental stress. Alternatively, the HOG pathway may act upon the same targets, regardless of the upstream signaling event, and circadian regulation may serve to prepare the organism for the daily exposure to hyperosmotic conditions that would be associated with desiccation by the sun in a natural environment. We have begun to address the issue of which of these two hypotheses is correct, and the preliminary data suggest that an accurate description will involve a combination of these scenarios.

To begin to determine whether the HOG pathway serves two independent functions (osmotic response and circadian output) or one clock-controlled function (circadian-regulated, osmotic-stress response), we examined induction of phospho-OS-2 and *ccg-1* mRNA levels in response to 4% NaCl at DD12 and DD24 in the wild-type strain. Recall

that at DD12 the levels of *ccg-1* mRNA and phospho-OS-2 are at their peak, whereas at DD24 these levels are at their trough (Figures 3-2A and 3-4B). If circadian regulation of the HOG pathway is in fact preparatory, we would expect that addition of 4% NaCl at DD12, when the HOG pathway is already activated by the clock, would result in a more modest response (*i.e.,* lower level of induction of phospho-OS-2 and *ccg-1* mRNA), than the same stimulus at DD24, when this pathway is less active and an acute response must be mounted in order to adapt to the hyperosmotic conditions. If the circadian regulation of this pathway is completely separate from the osmotic regulation, and is not preparatory for hyperosmotic conditions, we would expect a similar response to osmotic stress at both times of the day.

We found that phospho-OS-2 levels are rapidly induced to high levels within one minute of exposure to 4% NaCl at both DD12 and DD24 (Figure 4-2A). At each time point, induced phospho-OS-2 levels reach a peak between two and five minutes of induction and then begin to decline, but remain elevated, for at least one hour in the presence of 4% NaCl. As expected, pre- and mock-induced phospho-OS-2 levels are higher at DD12 than at DD24, reflecting the clock-controlled regulation in the absence of hyperosmotic stress (Figure 4-2A). These data suggest that in spite of the circadian regulation of the HOG pathway, a substantial acute response must be mounted to these hyperosmotic conditions. However, *ccg-1* mRNA levels are induced less at DD12 (when they are already elevated) compared to DD24 (when they are low, Figure 4-2B) as would be predicted if circadian regulation of the HOG pathway was preparatory for this stress.

The difference in the level of induction of *ccg-1* mRNA at DD12 vs. DD24 does support the hypothesis that clock regulation of osmotically induced genes is such that, in these conditions, no further induction of downstream genes may be necessary. However, the levels of phospho-OS-2 are highly induced at both times of day, which is suggestive of a more complicated scenario. At this time we cannot explain why the induction of phospho-OS-2 levels at DD12 does not result in a commensurate increase in *ccg-1* mRNA levels, as is observed at DD24. This experiment has only been performed one time and needs to be repeated before the results can be fully interpreted; however, notably there is a considerable time-lag between the induction of phospho-OS-2, which occurs within one minute of exposure to 4% NaCl (Figure 4-2A), and the induction of *ccg-1* mRNA, which occurs between 30 and 60 minutes post-induction (Figures 3-6B and 4-2B). This time lag, and the differential induction of these two species at DD12 (Figure 4-2), imply that the indirect regulation of *ccg-1* mRNA by phospho-OS-2 likely involves multiple steps, and that these steps may be regulated independently of one another such that elevated levels of phospho-OS-2 do not necessarily lead to an increase in *ccg-1* transcript levels. This suggests the possibility of preparatory regulation of this pathway by the clock that is slightly different than the regulation by osmotic stress. The organism may be primed to better respond to osmotic stress at a time of day when this response is more likely to be required (daytime) versus at other times of day when desiccation is less likely to occur (nighttime), but is able to mount an acute response, if required, at any time of day. Additionally, the over-activation of the HOG pathway in the absence of hyperosmotic

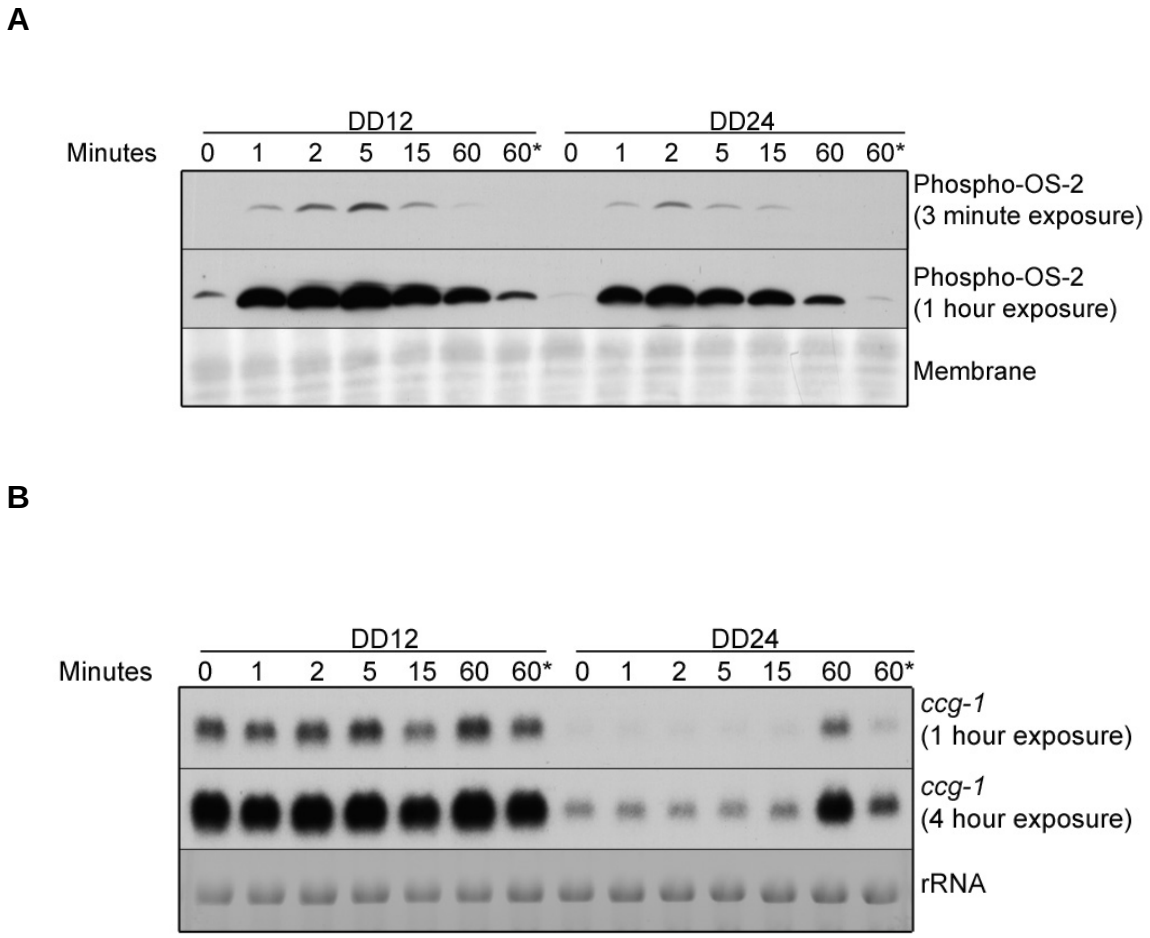


Figure 4-2. Circadian regulation of the HOG pathway results in different levels of response to osmotic stress at different times of day**.** (A) Western blot showing levels of phospho-OS-2, and (B) Northern blot showing levels of *ccg-1*, in the presence of 4% NaCl after 12 and 24 hours in DD. In both panels, hours in DD and minutes in 4% NaCl are given at the top, an asterisk indicates a control treatment with water instead of NaCl; exposure times are shown to the right. The Amido Black stained membrane (A) and ethidium bromide stained gel (B) are shown as loading controls.

conditions is lethal, causing cells to swell until they burst (Zhang et al., 2002). It may be that the clock regulation of the HOG pathway results in the activation of only a subset of the components involved in the osmotic-stress response. By readying some aspects of this pathway without activating the entire stress response, the cells could better prepare for predictable daily hyperosmotic conditions, without being subjected to the detrimental effects of an inappropriate osmotic-stress response. The identification of the point at which the clock impinges on the HOG pathway, in addition to the further characterization of the relationship between the circadian and osmotic control over this pathway, will no doubt help to shed light on this complex regulatory machinery.

A Model of Circadian Output

The preliminary characterization of the COP mutants in Chapter II suggested that regulation of *ccg-1* and *ccg-2* is though a single output pathway from the clock, which forks at some point to differentially regulate the expression of these two genes. The results described in Chapter III allow us to begin to fill in some of the missing pieces in the model of circadian output proposed in Chapter II. Although verification of the involvement of the HOG pathway in circadian regulation of *ccg-2* has not been extensively carried out, the data concerning *ccg-1* suggest that our original model may be correct. The results suggest that WC-1 is involved in inducing the levels of phospho-OS-2 by some as yet undescribed mechanism: phospho-OS-2 is undetectable in a Δ*wc-1* strain in the absence of osmotic stress (Figure 3-5), which results in low *ccg-1* mRNA levels (Figure 3-2A). Phospho-OS-2 activates downstream effectors that, in turn,

differentially regulate the expression of *ccg-1* and *ccg-2*. FRQ acts to inhibit the activation of phospho-OS-2 levels by the WC-1: phospho-OS-2 levels are constitutively elevated in a Δ*frq* strain (Figure 3-5), which results in high *ccg-1* mRNA levels. The further characterization of *ccg-2* expression in the Δ*rrg-1* strain, and the description of the additional factors that lie between the clock and the HOG pathway, and between the HOG pathway and downstream ccgs, will allow more of the gaps in this model to be filled in.

Circadian Output Pathways: Do They Exist?

Clock-controlled genes have traditionally been defined as those genes that display circadian-regulated expression but are not involved in, or required for, normal rhythmicity of the central oscillator. What is not expressly stated by this second criterion, but has been demonstrated through microarray analyses, is that ccgs function in nearly all other aspects of the cell's biology that are unrelated to clock function. This global involvement of ccgs in cellular processes implies a role for the clock as a 'master regulator' of the organism. For each well-characterized ccg, at least two different levels of regulation have been demonstrated: regulation by the clock, and regulation by the other components of whichever process in which the ccg is involved. For example, in *N. crassa, ccg-1* (unknown function) is regulated by the clock, developmental cues, and in response to osmotic stress and heat-shock (Chapter III; Lindgren, 1994); *ccg-9* (trehalose synthetase) is regulated by the clock and osmotic stress, (Shinohara et al., 2002); *ccg-2*, which encodes the hydrophin that maintains the hydrophobicity of conidia, is regulated

by the clock and developmental cues (Bell-Pedersen et al., 1996a). These examples raise the question of how a master regulator is able to influence the activity of such a multitude of processes that are each independently regulated by some other means.

Earlier studies investigating the relationship between clock and developmental regulation of *ccg-1* and *ccg-2* found that these two signals do not overlap (Correa and Bell-Pedersen, 2002). Circadian regulation remains intact in mutant strains that have lost the developmental regulation of these genes. These findings suggested that the clock-control of *ccg-1* and *ccg-2* is not merely a result of circadian influence over the developmental pathway, but that clock-specific output pathways may exist that are responsible for the circadian regulation of these genes. However, it is important to note that circadian regulation of the developmental pathway was also demonstrated in this study (Correa and Bell-Pedersen, 2002). The data presented in Chapter III can help explain these findings, and suggest that clock-specific output pathways may not be necessary if the clock regulates the activity of 'master-regulatory pathways', (for example, MAP kinase cascades).

We have now demonstrated that circadian regulation of *ccg-1* occurs through the osmotic-stress responsive HOG MAP kinase pathway, which is consistent with the developmental pathway not being involved in the circadian regulation of this gene. Although it has not yet been investigated in the Δ*rrg-1* strain, the initial characterization of the COP1-4 mutant strain revealed altered circadian control of *ccg-2* expression as

well as *ccg-1* (Chapter II), which indicates that the HOG pathway may also contribute to the overall rhythmicity observed in *ccg-2* mRNA levels and is also consistent with the previous study (Correa and Bell-Pedersen, 2002). As discussed earlier, the HOG pathway may have separable and specific functions with regard to stress response and the clock; however, it is not a clock-specific pathway as predicted, because it is common to both regulatory systems. In this sense, clock-specific output pathways may not actually exist; rather clock control may be exerted through the regulation of 'high-impact' regulatory pathways in place for other forms of cellular control.

Concluding Remarks

There are still many questions to be explored with regard to circadian output. The clock regulation of 'master-regulatory pathways' provides a mechanism for clock-control of a broad range of processes. These findings provide a valuable clue as to where to search for additional pathways involved in circadian output; however, the question of how the clock impacts these regulatory pathways remains unanswered at this time. In *N. crassa*, the core oscillator component FRQ has been shown to recruit kinases to the WCC as part of its function in the oscillator (He et al., 2006). Aside from its role as a central oscillator component, there have been no other functions ascribed to the FRQ protein. It is plausible that some time-of-day information from the clock is signaled directly through interaction of FRQ with kinases that are involved in the regulation of output (MAP kinase) pathways.

The question of what exactly the HOG pathway is regulating to confer circadian rhythmicity to downstream genes also needs to be addressed. Examination of transcription factors in *N. crassa* that are homologous to those under control of the *S. cerevisea* HOG pathway may yield some insight in this area. Interestingly, a connection between stress-related MAP kinase pathways and chromatin modifications, including phosphorylation and acetylation, has been established in mammals (for review see Clayton and Mahadevan, 2003). In addition, it was recently discovered that the mammalian CLOCK protein is a histone acetyl transferase (Doi et al., 2006), suggesting a role for chromatin modification in clock regulation of gene expression. This suggests the possibility that clock-regulated MAP kinase activity may affect gene expression through chromatin remodeling, and that this mechanism may be conserved from fungi to humans.

The more that is learned of circadian clock systems, the more it becomes apparent what an integral part of nearly every organism's basic biological processes they are. The work presented in this dissertation suggests that a different perspective may be helpful when considering the clock and clock-regulated processes. For simplicity's sake, we often try to think of the clock as a system that is separable from and independent of the processes which it is regulating. It is important to remember that the clock is involved in the control of most cellular processes, right down to general cellular metabolism, which means that by definition, the clock is intertwined with, and a part of, the very processes it regulates.

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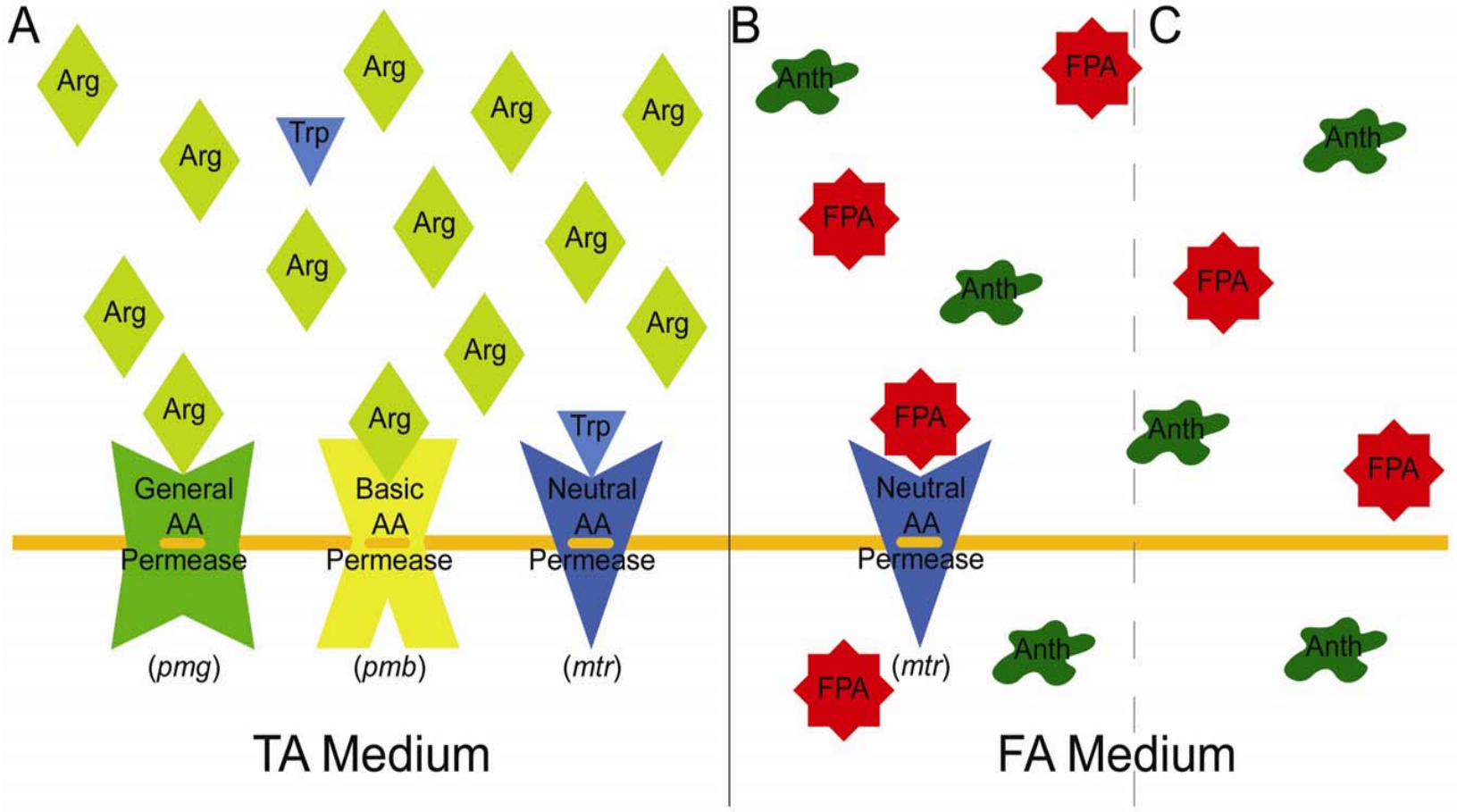
APPENDIX A

The mtr *Selection Scheme*.



A) Growth on TA medium requires expression of *mtr*, and B) expression of *mtr* inhibits growth on medium containing FPA (strain CCG1M pre-mutagenesis and CCG2M post-mutagenesis; see text for details). C) Growth can occur in the presence of FPA when *mtr* is not expressed (strain CCG2M pre-mutagenesis and CCG1M post-mutagenesis; see text for details)

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